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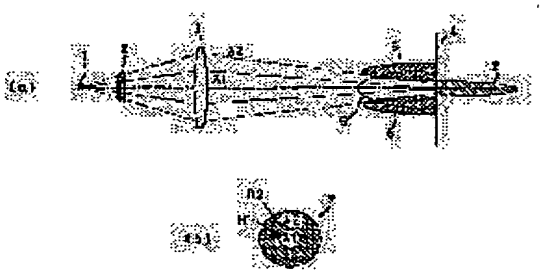
(54) OBSERVING METHOD OF MICROSCOPE

(57)Abstract:
PROBLEM TO BE SOLVED: To provide an observing method for obtaining a microscopic image of high spacial resolving power by using a molecule suited to the principle of a double resonance absorption process for dyeing a sample as an object to be observed and irradiating the dyed sample with light having plural wavelengths with good timing in the observation of a fluorescent microscope.
SOLUTION: In this observing method, light having a wavelength λ1 is converged on the surface 4 of a sample and the surface 4 of the sample is irradiated with light having a wavelength λ2 in a defocused state through an aperture 2 having a ring structure and an optical system 3 provided with a different converging position in the region of wavelengths λ1, λ2 from among light beams having wavelengths λ1, λ2 emitted from a light source 1. Consequently, the spacial resolving power of the microscope is improved beyond the condition of diffraction limit by the wavelength.



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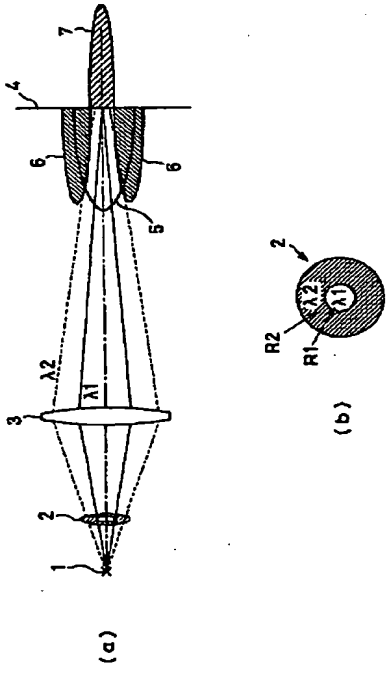
(54)【発明の名称】　顕微鏡の観察方法

(57)【要約】
【課題】　蛍光顕微鏡の観察において、観察目的物である試料の染色に二重共鳴吸収過程の原理にかなう分子を用い、染色された試料に複数の波長の光を良好なタイミングで照射することにより、高い空間分解能の顕微鏡像を得る観察方法を提供する。
【解決手段】　本発明の観察方法では、光源１から発せられた波長λ_1，λ_2の光を、輪帯構造を有するアパーチャー２及びλ_1とλ_2の波長帯域で異なる集光位置を備えた光学系３を介し、波長λ_1の光は試料面４上に集光させ、波長λ_2の光はデフォーカスされた状態で試料面４を照射させる。このようにすることにより、波長による回折限界条件を越えて顕微鏡の空間分解能を向上させることができる。



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【特許請求の範囲】
【請求項１】　試料の染色のために少なくとも基底状態を含め３つの量子状態を有する分子を用い、
該分子を第１励起状態へ励起させる波長λ_1の光を発する光源と、第１励起状態の分子を第２励起状態又はこれより高い励起状態へ励起させる波長λ_2の光を発する光源と、前記波長λ_1の光と波長λ_2の光を前記試料上に集光させる集光光学系と、励起された前記試料を染色した分子が基底状態へ脱励起する際の発光を検出する発光検出器と、前記波長λ_1の光の照射領域と前記波長λ_2の光の照射領域とを一部重ね合わせる重ね手段と、を備え、
該重ね手段を通して前記波長λ_1の光と波長λ_2の光とを前記試料に照射することにより、第１励起状態から基底状態へ脱励起する際の発光の領域を抑制するようにした光学顕微鏡において、
前記試料を染色する分子として、第１励起状態を除く高位のエネルギー状態から基底状態へ脱励起するときの遷移が蛍光による緩和過程よりも熱緩和過程が支配的である分子を蛍光プローブ分子として用いるようにしたことを特徴とする顕微鏡の観察方法。
【請求項２】　独立又は従属的に前記波長λ_1の光及び波長λ_2の光の偏光状態を可変し得るようにした請求項１に記載の顕微鏡の観察方法。
【請求項３】　前記試料に対する前記波長λ_1の光及び波長λ_2の光の照射時間を該試料を染色する分子の蛍光寿命の１０分の１とした請求項１又は２に記載の顕微鏡の観察方法。
【発明の詳細な説明】
【０００１】
【発明の属する技術分野】本発明は、光学顕微鏡を用いた観察方法に関する。詳しくは、染色した試料を複数の波長の光により照明することで、高い空間分解能をもつ良質な画質を得ることを可能とした走査型蛍光顕微鏡を用いた観察方法に関するものである。
【０００２】
【従来の技術】光学顕微鏡の歴史は古く、現在まで様々なタイプの顕微鏡が開発されてきた。又、近年のレーザー技術及び電子画像技術をはじめとする周辺技術の進歩により、更に高機能の顕微鏡システムが開発されている。このような背景の中、例えば、特開平８－２４８２００号公報等により、複数の波長の光で試料を照明することによって発する二重共鳴吸収過程を利用し、得られる画像のコントラストの制御のみならず、化学分析をも可能とした高機能な顕微鏡が提案されている。以下、この顕微鏡を分光的な立場から説明する。
【０００３】図１１は、試料を構成する分子の価電子軌道の電子構造を示す図である。前記特開平８－２４８２００号公報に開示されている顕微鏡では、二重共鳴吸収過程を利用し、特定の分子を選択して、特定の光学遷移

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に起因する吸収及び蛍光を観察することができる。この原理を更に図１２乃至１４も用いて説明する。
【０００４】まず、図１１に示す基底状態（以下、Ｓ。状態と称する）の分子がもつ価電子軌道の電子を、図１２に示すようにある波長（λ_1）の光により励起する（第１励起状態：以下、S_1状態と称する）。次に、同じく図１３に示すように、別の波長（λ_2）の光により励起する（第２励起状態という：以下、S_2状態と称する）。このS_2状態になった分子は、この後図１４に示すように、蛍光又は燐光を発光したりしてS_0状態に戻る。二重共鳴吸収過程を利用した顕微鏡による観察方法は、図１２，１３に示された吸収過程や図１４に示された蛍光や燐光の発光を用いて、吸収像や発光像を観察するものである。
【０００５】この観察方法では、まず最初に、レーザー光等の共鳴波長λ_1の光を用いて、試料を構成する分子を図１２に示されたようなS_1状態に励起させるが、S_1状態における単位体積内の分子数は照射する光の強度が増加するに従い増加する。又、線吸収係数は、分子１個当たりの吸収断面積と単位体積当たりの分子数との積で与えられる。よって、図１３に示すような励起状態において、最初に照射された波長λ_1の光に続いて照射される共鳴波長λ_2に対する線吸収係数は、前記波長λ_1の光の強度に依存する。即ち、波長λ_2の光に対する線吸収係数は、波長λ_1の光の強度で制御できることになる。このことは、波長λ_1と波長λ_2の２波長の光で試料を照射し、波長λ_2の光による透過像を撮影すれば、透過像のコントラストは波長λ_1の光量で完全に制御できることを示している。
【０００６】一方、図１４に示されたように、S_2状態から蛍光又は燐光による脱励起状態が可能である場合には、その発光強度はS_1状態にある分子の数に比例する。従って、蛍光顕微鏡として利用する場合にも画像コントラストの制御が可能となる。又、この方法はコントラストの制御のみならず化学分析も可能としている。
【０００７】ここで、図１１に示された最外殻価電子軌道は、各々の分子に固有なエネルギー準位を有する。従って、波長λ_1の光を照射した場合の蛍光は、分子によって異なる。同時に、波長λ_2の光を照射した場合も各分子固有の蛍光を発する。従来のように、単一波長の光を試料に照射して観察する場合であっても、ある程度特定の分子の吸収像又は蛍光像を観察することは可能である。しかし、一般には幾つかの分子の吸収帯の波長領域は重複するので、試料の化学組成の正確な同定までは不可能である。
【０００８】これに対して、二重共鳴吸収過程を利用した観察方法では、波長λ_1及び波長λ_2の２波長の光により吸収又は発光する分子を限定するので、従来の方法よりも正確な試料の化学組成の同定が可能となる。又、価電子を励起する場合、分子軸に対して特定の電場ベク

トルをもつ光のみが強く吸収されるので、波長λ_1及び波長λ_2の偏光方向を定めて吸収又は蛍光像を撮影すれば、同じ分子でも配向方向の同定まで可能になる。

【０００９】更に、最近では、二重共鳴吸収過程を利用した解析限界を越える高い空間分解能をもつ蛍光顕微鏡が提案されている。この顕微鏡の原理を図１５を用いて説明する。図１５はある分子における二重共鳴吸収過程を示す概念図である。S_0状態にある分子が、波長λ_1の光でS_1状態に励起され、更に波長λ_2の光でS_2状態に励起される様子を示している。そして、この分子のS_2状態における蛍光が極めて弱いことを示している。

【００１０】ところで、このような光学的性質を有する分子に対して、極めて興味深い現象が期待できる。これを図１６を用いて説明する。図１６は、図１５と同様、二重共鳴吸収過程の概念図であるが、横軸にＸ軸を設け空間距離の拡がりを表現している。そして、波長λ_2の光を照射される空間領域A_1と、波長λ_2の光が照射されない空間領域A_0が示されている。

【００１１】空間領域A_0では波長λ_1の光による励起でS_1状態の分子が多数生成される。このとき、空間領域A_0からは波長λ_1の蛍光の発光がみられる。しかし、空間領域A_1では波長λ_2の光を照射したため、S_1状態の分子が殆ど即座により高位のS_2状態に励起され、S_1状態の分子は存在しなくなる。かかる現象は、幾つかの分子により確認されている。この現象により、波長λ_1の蛍光は完全になくなり、しかも、S_2状態からの蛍光はもともと存在しないため、空間領域A_1では完全に蛍光自体が抑制される。従って、空間領域で蛍光が存在するのはA_0領域のみであることが分かる。

【００１２】このような結果は、顕微鏡分野において極めて重要な意味をもつ。即ち、従来の走査型レーザー顕微鏡等では、レーザー光を集光してマイクロビームを形成して観察試料上を走査する。このとき、マイクロビームの照射領域は集光レンズの開口数と波長で定まる回折限界で定まり、原理的にそれ以上の空間分解能は期待できない。しかし、図１６に示されたように、波長λ_1，λ_2の２種類の波長を空間的に上手く重ね合わせることで、前述のように波長λ_2の光の照射で蛍光領域が抑制される。このとき、例えば波長λ_1の光の照射領域に着目すると、蛍光領域は集光レンズの開口数と波長で定まる波長λ_1の照射領域よりも狭くなっており、実質的に空間分解能の向上が計られる。従って、かかる原理を用いれば回折限界を越える蛍光顕微鏡を実現できる（二重共鳴吸収過程を利用した超解像顕微鏡）。

【００１３】

【発明が解決しようとする課題】ところで、蛍光顕微鏡では、通常、蛍光ラベラーと呼ばれる特殊な分子で試料（主に生体細胞）を染色して観察が行われる。この蛍光ラベラーは、特定の波長の光を吸収すると、比較的強い蛍光を発する。そこで、前述の観察方法にこの蛍光ラベラーを用いれば大変効果的であると考えられるが、実際に蛍光顕微鏡での観察に応用された例としては、トリプトファンとアデニンをはじめとするごく一部の分子についてのみその有効性が示されているにすぎない。尚、このトリプトファンとアデニンは、生体細胞に組み込まれているアミノ酸分子又は核酸塩基であり、細胞内にあって自家蛍光を発する特殊な分子である。

【００１４】実際、無染色で観察できる分子は種類が限られており、多くの種類の分子は無染色ではその組成を観察することはできない。蛍光ラベラーは、その官能基を選択することで観察目的の特定の分子のみを染色できるという優れた特性がある。二重共鳴吸収過程を利用した超解像顕微鏡に適応し、その機能を十分に活かすためには、前述の原理に適した蛍光ラベラー分子を選定し、しかも利用する波長λ_1及び波長λ_2の２つの光の試料への照射タイミングを検討する必要がある。しかし、これまで、光を照射する適切なタイミングと、蛍光ラベラー分子が具体的に示された例はない。

【００１５】そこで、かかる従来技術の問題点に鑑み、本発明は、蛍光顕微鏡の観察において、観察目的物である試料の染色に二重共鳴吸収過程の原理にかなう分子を用い、染色された試料に複数の波長の光を良好なタイミングで照射することにより、高い空間分解能の顕微鏡像を得る観察方法を提供することを目的とする。

【００１６】

【課題を解決するための手段】上記目的を達成するため、本発明による顕微鏡の観察方法は、試料の染色のために少なくとも基底状態を含め３つの量子状態を有する分子を用い、この分子を第１励起状態へ励起させる波長λ_1の光を発する光源と、第１励起状態の分子を第２励起状態又はこれより高い励起状態へ励起させる波長λ_2の光を発する光源と、前記波長λ_1の光と波長λ_2の光を前記試料上に集光させる集光光学系と、励起された前記試料を染色した分子が基底状態へ脱励起する際の発光を検出する発光検出器と、前記波長λ_1の光の照射領域と前記波長λ_2の光の照射領域とを一部重ね合わせる重ね手段と、を備え、前記重ね手段を通して前記波長λ_1の光と波長λ_2の光とを前記試料に照射することにより、第１励起状態から基底状態へ脱励起する際の発光の領域を抑制するようにした光学顕微鏡において、前記試料を染色する分子として、第１励起状態を除く高位のエネルギー状態から基底状態へ脱励起するときの遷移が蛍光による緩和過程よりも熱緩和過程が支配的である分子を蛍光プローブ分子として用いるようにしたことを特徴とする。

【００１７】更に、本発明の顕微鏡の観察は、独立又は従属的に前記波長λ_1の光及び波長λ_2の光の偏光状態を可変し得るようにしたことを特徴とする。又、前記試料に対する前記波長λ_1の光及び波長λ_2の光の照射時間を前記試料を染色する分子の蛍光寿命の１０分の１と

したことも特徴としている。

【００１８】

【発明の実施の形態】本発明では、少なくとも基底状態を含め３つの量子状態を有しており、第１励起状態を除く高位のエネルギー状態から基底状態へ脱励起するときの遷移が蛍光による緩和過程よりも熱緩和過程が支配的である分子を用いて試料の染色を行うことを基本とする。そして、この種の分子を用いることで、二重共鳴吸収過程を利用した超解像顕微鏡での観察を可能にしている。この分子を用いて生化学的な染色技術を施した生体試料に、波長λ_1の光を照射することによりS。状態からS_1状態に励起し、続いて即座に波長λ_2の光により高位な量子準位に励起することで、S_1状態で発せられる蛍光を抑制するものである。本発明は、このような分子の光学的な性質を利用し、空間的な蛍光領域を人為的に抑制し、空間分解能の向上を図るものである。以下、かかる分子の光学的な性質を量子化学的な立場から説明する。

【００１９】一般に、分子を構成する各原子はσ又はπ結合によって結ばれている。例えば、三共出版「量子化学材料入門(1991)（足立裕彦）」によれば、分子の分子軌道はσ分子軌道又はπ分子軌道をもっており、これらの分子軌道に存在する電子が各原子を結合する重要な役割を担っている。なかでも、σ分子軌道の電子は、各原子を強く結合し分子の骨格である分子内の原子間距離を決定している。

【００２０】これに対して、π分子軌道の電子は、各原子結合には殆ど寄与せず、寧ろ分子全体に極めて弱い力で束縛される。多くの場合、σ分子軌道にある電子を光で励起すると、分子の原子間隔が大きく変化し、分子の解離を含む大きな構造変化が生じる。その結果、原子の運動エネルギーや構造変化するため光が分子に与えたエネルギーの殆どが熱エネルギーに形を変える。従って、励起エネルギーは蛍光という光の形態では消費されない。又、分子の構造変化は極めて高速に起きるので（ピコsec より短い時間）、その過程で仮に蛍光が生じても極めて蛍光寿命が短いものとなる。

【００２１】しかし、これに対し、π分子軌道の電子は励起しても、分子の構造自体は殆ど変化せず高位の量子的な解離準位に長時間止まり、nsecのオーダーで蛍光を放出し脱励起する性質をもつ。量子化学によれば、分子がπ分子軌道をもつことと、二重結合をもつこととは同等であり、本発明で用いる蛍光ラベラー分子には、二重結合を豊富に有する分子を選定することが必要条件となる。

【００２２】更に、二重結合をもつ分子でも、ベンゼンやピラジン等の６員環分子においては、S_2状態からの蛍光が極めて弱いことが知られている（M.Fujii et.al. Chem.Phys.Lett.171(1990)341)。従って、ベンゼンやピラジン等の６員環分子を含む分子を蛍光ラベラーとして

選定すれば、S_1状態からの蛍光の寿命は長く、しかも光励起によりS_1状態からS_2状態へ励起することで分子から生じる蛍光を容易に抑制できるので、本発明の方法を効果的に行える。即ち、これらの蛍光ラベラー分子により染色して観察を行えば、高空間分解能で試料の蛍光像を観察することができるのみならず、その分子の側鎖の化学基を調整することにより生体試料の特定の化学組織のみを選択的に染色できるので、詳細な試料の化学組成までも分析できる。

【００２３】一般に、二重共鳴吸収過程は、２つの光の波長や偏光状態等が特定の条件を満たすときに起きるので、これを用いることで非常に詳細な分子の構造を知ることができる。即ち、光の偏光面と分子の配向方向とには強い相関関係があり、２つの波長の光夫々の偏光面と分子の配向方向とが特定の角度をなすとき、二重共鳴吸収過程が強く起きる。従って、２つの波長の光を試料面に向けて同時に照射し、夫々の光の偏光面を回転させることにより、蛍光の消失の程度が変化するので、この様子を観察すれば試料の組織の空間配向の情報も得られる。更に、かかる偏光面を回転させずに、照射する２つの光の波長を調整することでも、試料の組織の空間配向を知ることはできる。

【００２４】以上のように、本発明の方法によれば、超解像性以外にも高い分析能力が得られることが分かる。

【００２５】更に、本発明では、二重共鳴吸収過程を利用した超解像顕微鏡を用いる観察方法において、蛍光抑制を効果的に発生させることが可能な蛍光ラベラー分子の選定と、適正なタイミングで光を試料に照射する方法も提案する。この原理を、図１を用いて説明する。

【００２６】二重共鳴吸収過程を利用した超解像顕微鏡による観察法は、波長λ_1の光により蛍光ラベラー分子を励起し、波長λ_2の光でその蛍光を消すことを基本としている。図１は、λ_1，λ_2の２種類の波長の光を試料に照射するタイミングを示している。この図によれば、試料へ向けてまず時間ｔだけ波長λ_1の光を照射し、引き続き波長λ_2の光を照射しているが、このとき各波長の光の照射時間を蛍光ラベラー分子の蛍光を発する時間、即ちS_1状態の寿命より短くしている。定性的に述べると、まず波長λ_1の光を蛍光ラベラー分子のS_1状態の寿命より十分短い時間ｔの間照射し、観察領域にS_1状態の分子を生成させる。その直後に、観察に不要な領域に同じくS_1状態の寿命より十分短い時間波長λ_2の光を照射し、S_1状態にある分子をS_2状態に励起し、蛍光を抑制する。以下、この過程を定量的に説明する。

【００２７】一般に、S。状態の分子を波長λ_1の光でS_1状態に励起する場合、その励起過程は以下に示すレート方程式により記述できる。即ち、試料を染色した分子の単位体積当たりの分子数をN。とし、波長λ_1の光のフォトンフラックスをI_0、波長λ_1の光の照射より

時間ｔ後のＳ。状態の分子数をＮとする。そして、Ｓ₁状態の寿命をτとし、波長λ₁の光によりＳ。状態からＳ₁状態に遷移するときの吸収断面積をσ_{01}とすると、このレート方程式は次の式（１）のように具体的に表すことができる。

$$-\frac{dN}{dt}=NI_0\sigma_{01}-\frac{(N_0-N)}{\tau} \quad \cdots\cdots(1)$$

【００２８】更に、この式（１）を解けば、波長λ₁の光の照射より時間ｔ後の単位体積当たりのＳ₁状態の分子数ｎを求めることができる。

$$n=\frac{N_0I_0\sigma_{01}\tau}{(1+I_0\sigma_{01}\tau)}\cdot\left[1-e^{\left\{-(I_0\sigma_{01}+\frac{1}{\tau})t\right\}}\right] \quad \cdots\cdots(2)$$

但し、$n=N_0-N$

【００２９】又、式（２）を満たす条件の下で、次の式（３）の条件を満足するように波長λ₁の光を照射すれば、前記式（２）は近似的に式（４）のように書ける。

$$(I_0\sigma_{01}+\frac{1}{\tau})t\cong 0 \quad \cdots\cdots(3)$$

$$n\cong I_0\sigma_{01}N_0t \quad \cdots\cdots(4)$$

【００３０】即ち、式（３）によれば、波長λ₁の光の照射時間を分子のＳ₁状態の寿命よりも短くし、しかも、波長λ₁の光のフォトンフラックスが小さいときは、Ｓ₁状態の分子数ｎは照射時間ｔにほぼ比例する。

【００３１】次に、波長λ₁の光の照射が終了し、その直後に波長λ₂の光を時間Ｔの間照射したときのＳ₁状態にある分子をＳ₂状態に励起する場合を考える。波長λ₂の光のフォトンフラックスをI_1、波長λ₁の光の照射より時間（Ｔ＋ｔ）後のＳ₁状態の分子数をｎとし、そして波長λ₂の光によりＳ₁状態からＳ₂状態に遷移するときの吸収断面積をσ_{12}とすると、ｎに関するレート方程式は次のようになる。

$$\frac{dn}{dt}=-\sigma_{12}I_1n-\frac{n}{\tau} \quad \cdots\cdots(5)$$

【００３２】この式（５）を解くことで、波長λ₁の光を時間ｔだけ照射した後に止め、その直後に波長λ₂の光を時間Ｔの間照射した場合の、前記ｎの値は次式（６）のように表すことができる。

$$n=(I_0\sigma_{01}N_0t)\cdot e^{-(\sigma_{12}I_1+\frac{1}{\tau})T} \quad \cdots\cdots(6)$$

一方、式（６）において、波長λ₂の光を全く照射しない場合には$I_1=0$として、

$$n=(I_0\sigma_{01}N_0t)\cdot e^{\frac{T}{\tau}} \quad \cdots\cdots(7)$$

となる。

【００３３】実は、式（６）は蛍光を抑制された領域における単位体積当たりのＳ₁状態の分子数を示し、式（７）は蛍光を抑制されない領域における単位体積当たりのＳ₁状態の分子数を示している。分子の蛍光吸収率をΦとすると、蛍光を抑制された領域からの蛍光強度Ｆ

₁と、蛍光を抑制されない領域からの蛍光強度Ｆ₂とは、夫々次の式により与えられる。

$$F_1=\Phi(I_0\sigma_{01}N_0t)\cdot e^{-(\sigma_{12}I_1+\frac{1}{\tau})T} \quad \cdots\cdots(8)$$

$$F_2=\Phi(I_0\sigma_{01}N_0t)\cdot e^{\frac{T}{\tau}} \quad \cdots\cdots(9)$$

【００３４】又、蛍光抑制比率F_1/F_2は、式（８），（９）により次式のように表される。

$$\frac{F_1}{F_2}=e^{-\sigma_{12}I_1T} \quad \cdots\cdots(10)$$

【００３５】従って、図１に示されるタイミングでλ₁とλ₂の２種類の波長の光を照射すれば、式（１０）により示される比率で観察に必要としない領域からの蛍光を抑制できる。式（１０）によれば、Ｔ＜τの条件でI_1とＴとを調整することにより、任意の比率で蛍光を抑制することができる。

【００３６】図２には、観察領域からの蛍光強度を観測するタイミングが示されている。蛍光強度を測定するタイミングは、基本的には、波長λ₂の光の照射が終了した後に、観察領域から発光する蛍光の強度を十分に時間をかけて測定することになる。このようにすれば、抑制された領域からの蛍光が殆ど存在しない状態で、即ち非常によいＳ／Ｎ比の状態で観察領域からの蛍光を測定することができる。

【００３７】図３及び４も、λ₁とλ₂の２種類の波長の光を試料に照射するタイミングと、観察領域から発光される蛍光の強度を測定するタイミングを示す図である。ここに示されたタイミングで行っても、効果的に蛍光の測定ができる。しかし、図２乃至４示された何れの場合でも、ｔ，Ｔ＜τの条件を満足することが必要がある。なぜなら、ｔ，Ｔ＞τであると、λ₁とλ₂の２種類の波長の光を照射中にＳ₁状態の分子がＳ。状態に脱励起してしまい、観察領域からの蛍光自体がなくなってしまうためである。仮に、ｔ，Ｔ＞τの場合に対応するために、図５に示すように、λ₁とλ₂の２種類の波長の光を同時に照射して、同時に観察領域から発光する蛍光の強度を測定する方法も可能であるが、この場合には、蛍光測定時にλ₁とλ₂の２種類の波長の強い励起光が検出器に混入してしまう虞がある。従って、ｔ，Ｔ＜τの条件の下で、図２乃至４に示されたタイミングでλ₁とλ₂の２種類の波長の光を試料に照射するのが望ましい。

【００３８】又、本発明では、λ₁とλ₂の２種類の波長の光の試料への照射終了直後から、観察領域から発光する蛍光を検出器で測定しなくてはならないが、その際、市販の汎用ロジック回路によりゲート信号を生成し、検出器からの出力される電気信号をパソコンのメモリーに取り込む作業が必要になる。このとき、図１乃至４に示されたタイムチャートのように、本発明の方法を実行する際には、試料に対する光の照射時間が、試料を

染色する分子の蛍光寿命よりも短くなるようにすれば効果的である。しかし、現在市販されている汎用ロジック回路では、そのスイッチング速度が１nsec程度であるため、τ自体は１nsec以上であることが望まれる。なぜなら、τが１nsec以上でないと検出器及び計測回路がアクティブになる前に、観察領域からの蛍光現象が終了してしまうからである（日本テキサスインスツルメント株式会社：Texas Instruments ALS/AS アドバンスドバイポーラロジックファミリーデータブック(1991)）。以上の事情を考慮して、試料を染色する蛍光ラベラー分子には、１nsec以上の蛍光寿命をもっていなくてはならないという条件が付加されることになる。

【００３９】更に、測定データを抽出するための有効蛍光領域に着目すると、確かに蛍光抑制領域では蛍光強度が弱いほうが好ましいが、Ｓ／Ｎ比の向上という観点からは、有効蛍光領域の発光強度は強いほうが望ましい。即ち、波長λ_1の光で励起された直後のS_1状態の分子数が十分存在している時刻における蛍光強度を測定することが望ましい。前記式（９）によれば、励起分子の数はその励起寿命で定まる時定数により指数関数的に減衰する。

【００４０】ところで、指数関数の性質として、光の照射時間ｔ，ＴがS_1状態の分子の蛍光寿命τより十分短ければ、波長λ_1の光で励起された直後のS_1状態の分子から十分強い強度の蛍光、即ち有効信号強度を測定できる。特に、ｔ，ＴがS_1状態の分子の蛍光寿命τの１０分の１程度であれば、S_1状態の分子数は波長λ_1の光で励起された直後の分子数の９０％もあるので、有効蛍光領域から十分な信号強度が得られる。

【００４１】以下、図示した実施例に基づき本発明を詳細に説明する。

【００４２】第１実施例

ε－アデノシン(1,N^6-ethenoadenosine)は代表的な蛍光ラベラーであり、本実施例では、この分子を蛍光プローブ分子として用いた走査型蛍光顕微鏡による観察方法を紹介する。

【００４３】ε－アデノシンは、ヌクレチオの形（ε-ATP，ε-ADP）でＡＴＰやＡＤＰの分布量を示す良質なラベラー分子として働くことが、多くの酵素系で証明されている。一方、蛍光プローブ分子として考えると、蛍光収率が高いこと、励起波長が核酸や蛋白質と重ならないこと等が、生体試料に応用するうえで利点となる。更に、蛍光波長や蛍光収率は、溶媒の極性等によって変化を受けないにもかかわらず、蛍光の極大波長が大きくなるにつれて、短波長側に移動する性質を有している（学会出版センター「蛍光測定(1933)大下一彦／御橋廣眞」）。このため、ε－アデノシンを蛍光プローブ分子として蛋白質等に応用する場合に、蛍光発光波長がヌクレチオド結合部位の束縛度や、環境の粘性の指標となり得ることを示している。

【００４４】以上の点を活かして、ε－アデノシンは、蛍光スペクトルの変化に基づく酵素の構造変化、エネルギー移動の観測に基づくアクチンやミオシンの活性中心付近の構造変化、偏光度や蛍光消失による緑葉体共役因子のヌクレチオド結合部位の識別等、多くの分野で利用可能である。又、ε－アデノシンは次の表１に示すような物理特性を有しており、特にS_1状態の励起寿命が極めて長く、ｐＨ７．０の水溶液中では、２０sec の蛍光寿命を有している。従って、ε－アデノシンを蛍光プローブ分子とし、これにより生体試料を染色し、二重共鳴吸収過程を利用した超解像顕微鏡を用いて観察を行えば、高空間分解能でヌクレチオド結合部位の詳細な化学組成分析が可能となる。以下、本実施例では、ε－アデノシンを蛍光プローブ分子とした場合について述べる。

【００４５】

表１　ε－アデノシンの物理特性

分子量	３２７．７３
溶解性／溶媒	水溶性
蛍光寿命	２０ｎｓｅｃ
蛍光収率	０．５６
最大吸収波長	２９４ｎｍ
最大蛍光波長	４１５ｎｍ

【００４６】まず、具体的な本実施例の観察方法を説明する前提として、この方法に用いられる二重共鳴吸収過程を利用した超解像顕微鏡の原理について説明する。図６（ａ）は、二重共鳴吸収過程を利用した超解像顕微鏡の主要構成要素であるマイクロビームを構成する光学系の構成を示す図である。ここでは、同じ点よりλ_1とλ_2の波長を有する光が分光されて発光される光源１を想定している。この光源１から発せられた波長λ_1，λ_2の光は、輪帯構造を有するアパーチャー２を照射する。アパーチャー２を抜けた光は、λ_1とλ_2の波長帯域で異なる集光位置を備えた光学系３により、波長λ_1の光は観察目的の試料面４上に集光され、波長λ_2の光はデフォーカスされた状態で試料面４上を照射する。このとき、試料は、光学系３の結像面内における二次元走査及び光軸方向の走査が可能なステージ上（不図示）に搭載されている。又、試料をマイクロビームで二次元走査する方法として、スキャンニングミラー等を用いてビーム自体を走査するようにしてもよい。

【００４７】又、ここで用いられる輪帯構造を有するアパーチャー２は次のような特別な構成を備えている。即ち、図６（ｂ）はアパーチャー２の構成を示す正面図であるが、アパーチャー２は領域R_1（中央部）と領域R_2（輪帯部）との二重輪帯構造になっている。二重輪帯構造のうち、領域R_1は波長λ_1の光に対して十分な透

染色する分子の蛍光寿命よりも短くなるようにすれば効果的である。しかし、現在市販されている汎用ロジック回路では、そのスイッチング速度が１nsec程度であるため、τ自体は１nsec以上であることが望まれる。なぜなら、τが１nsec以上でないと検出器及び計測回路がアクティブになる前に、観察領域からの蛍光現象が終了してしまうからである（日本テキサスインスツルメント株式会社：Texas Instruments ALS/AS アドバンスドバイポーラロジックファミリーデータブック(1991)）。以上の事情を考慮して、試料を染色する蛍光ラベラー分子には、１nsec以上の蛍光寿命をもっていなくてはならないという条件が付加されることになる。

【００３９】更に、測定データを抽出するための有効蛍光領域に着目すると、確かに蛍光抑制領域では蛍光強度が弱いほうが好ましいが、Ｓ／Ｎ比の向上という観点からは、有効蛍光領域の発光強度は強いほうが望ましい。即ち、波長λ_1の光で励起された直後のS_1状態の分子数が十分存在している時刻における蛍光強度を測定することが望ましい。前記式（９）によれば、励起分子の数はその励起寿命で定まる時定数により指数関数的に減衰する。

【００４０】ところで、指数関数の性質として、光の照射時間ｔ，ＴがS_1状態の分子の蛍光寿命τより十分短ければ、波長λ_1の光で励起された直後のS_1状態の分子から十分強い強度の蛍光、即ち有効信号強度を測定できる。特に、ｔ，ＴがS_1状態の分子の蛍光寿命τの１０分の１程度であれば、S_1状態の分子数は波長λ_1の光で励起された直後の分子数の９０％もあるので、有効蛍光領域から十分な信号強度が得られる。

【００４１】以下、図示した実施例に基づき本発明を詳細に説明する。

【００４２】第１実施例

ε－アデノシン(1,N^6-ethenoadenosine)は代表的な蛍光ラベラーであり、本実施例では、この分子を蛍光プローブ分子として用いた走査型蛍光顕微鏡による観察方法を紹介する。

【００４３】ε－アデノシンは、ヌクレチオの形（ε-ATP，ε-ADP）でＡＴＰやＡＤＰの分布量を示す良質なラベラー分子として働くことが、多くの酵素系で証明されている。一方、蛍光プローブ分子として考えると、蛍光収率が高いこと、励起波長が核酸や蛋白質と重ならないこと等が、生体試料に応用するうえで利点となる。更に、蛍光波長や蛍光収率は、溶媒の極性等によって変化を受けないにもかかわらず、蛍光の極大波長が大きくなるにつれて、短波長側に移動する性質を有している（学会出版センター「蛍光測定(1933)大下一彦／御橋廣眞」）。このため、ε－アデノシンを蛍光プローブ分子として蛋白質等に応用する場合に、蛍光発光波長がヌクレチオド結合部位の束縛度や、環境の粘性の指標となり得ることを示している。

【００４４】以上の点を活かして、ε－アデノシンは、蛍光スペクトルの変化に基づく酵素の構造変化、エネルギー移動の観測に基づくアクチンやミオシンの活性中心付近の構造変化、偏光度や蛍光消失による緑葉体共役因子のヌクレチオド結合部位の識別等、多くの分野で利用可能である。又、ε－アデノシンは次の表１に示すような物理特性を有しており、特にS_1状態の励起寿命が極めて長く、ｐＨ７．０の水溶液中では、２０sec の蛍光寿命を有している。従って、ε－アデノシンを蛍光プローブ分子とし、これにより生体試料を染色し、二重共鳴吸収過程を利用した超解像顕微鏡を用いて観察を行えば、高空間分解能でヌクレチオド結合部位の詳細な化学組成分析が可能となる。以下、本実施例では、ε－アデノシンを蛍光プローブ分子とした場合について述べる。

【００４５】

表１　ε－アデノシンの物理特性

分子量	３２７．７３
溶解性／溶媒	水溶性
蛍光寿命	２０ｎｓｅｃ
蛍光収率	０．５６
最大吸収波長	２９４ｎｍ
最大蛍光波長	４１５ｎｍ

【００４６】まず、具体的な本実施例の観察方法を説明する前提として、この方法に用いられる二重共鳴吸収過程を利用した超解像顕微鏡の原理について説明する。図６（ａ）は、二重共鳴吸収過程を利用した超解像顕微鏡の主要構成要素であるマイクロビームを構成する光学系の構成を示す図である。ここでは、同じ点よりλ_1とλ_2の波長を有する光が分光されて発光される光源１を想定している。この光源１から発せられた波長λ_1，λ_2の光は、輪帯構造を有するアパーチャー２を照射する。アパーチャー２を抜けた光は、λ_1とλ_2の波長帯域で異なる集光位置を備えた光学系３により、波長λ_1の光は観察目的の試料面４上に集光され、波長λ_2の光はデフォーカスされた状態で試料面４上を照射する。このとき、試料は、光学系３の結像面内における二次元走査及び光軸方向の走査が可能なステージ上（不図示）に搭載されている。又、試料をマイクロビームで二次元走査する方法として、スキャンニングミラー等を用いてビーム自体を走査するようにしてもよい。

【００４７】又、ここで用いられる輪帯構造を有するアパーチャー２は次のような特別な構成を備えている。即ち、図６（ｂ）はアパーチャー２の構成を示す正面図であるが、アパーチャー２は領域R_1（中央部）と領域R_2（輪帯部）との二重輪帯構造になっている。二重輪帯構造のうち、領域R_1は波長λ_1の光に対して十分な透

シンを用いた例を示したが、本実施例では、他の物質を蛍光プローブ分子として用いてもよい。例えば、以下に示す分子は、何れも６員環又は二重結合を含み、極めて長い蛍光寿命と大きい蛍光収率を有し、二重共鳴吸収過程を利用した超解像顕微鏡に適用可能なものである。

【００６４】4-Fluoro-7-sulfamoylbenzofurazan:3,6-Bis(dimethylamino)-10-dodecylacridinium bromide:4-Benzylamino-7-nitrobenzofurazan:4-Azidofluorescein diacetate,5,6-Dimethoxy-2-(4-hydrazinocarbonylphenyl)benzothiazole:3-Bromomethyl,6,7-dimethoxy-1-methyl-1,2-dihydroquinoxaline-2-one:4-Bromoethyl-7-methoxycoumarin:N-[4-(6-Dimethylamino-2-benzofuranyl]maleimide:1,2-Diamino-4,5-dimethoxybenzene,dihydrochloride:2,2'-Dihydroxy-6,6'-dinaphthyl disulfide:3-Chlorocarbonyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone:2-2'-Dithiobis(1-aminoaphthalene),Fluorescein-4-isothiocyanate:4-Amino-3-penten-2-one:4-(4-Methoxybenzylamino)-7-nitrobenzofurazan:N-[4-(5,6-Methylenedioxy-2-benzofuranyl)]maleimide:1,2-Diamino-4,5-methylenedioxybenzene:N-(9-Acridinyl)maleimide:4-Fluoro-7-nitrobenzofurazan,4-Chloro-7-nitrobenzofurazan:(S)-(-)-1-(2,3-Napthalenedicarboximidyl fluoride:2-(5-Chlorocarbonyl-2-oxazolyl)-5,6-methylenedioxybenzofuran:4-Chloro-7-sulfobenzofurazan,ammonium salt:4-Fluoro-7-sulfobenzofurazan,ammonium salt:Sulforthodamine 101 acid chloride:4-[4-(Dimethylamino)phenylazo]phenylisothiocyanate:3,5-Dinitrobenzoyl chloride:5-(4-Dimethylaminophenyl)-2,4-pentadienal:1,3-Diphenyl-2-thiobarbituric acid:O-(4-Nitrobenzyl)-N,N'-diisopropylisourea:O-(4-Nitrobenzyl)hydroxylamine,hydrochloride:N,N,N'-Triethyl-N'-[N-(N-succinimidyloxycarbonyl)pentyl]-9-cyanopyronine chloride:N,N,N'-Triethyl-N'-｛5-[N''-(2-maleimidoethyl)piperazinocarbonyl]pentyl｝9-cyanopyronine chloride:2,4'-Dibromoacetophenone:N-Succinimidyl-4-nitrophenylacetate:

【００６５】O,O'-Bis(2-aminophenyl)ethyleneglycol-N,N,N',N'-tetraacetic acid,tetraapotassium,hydrate:O,O'-Bis(2-aminophenyl)ethyleneglycol-N,N,N',N'-tetraacetic acid,tetraacetoxymethyl ester:O,O'-Bis(2-amino-5-fluorophenoxy)ethyleneglycol-N,N,N',N'-tetraacetic acid,tetraapotassium,hydrate:O,O'-Bis(2-amino-5-fluorophenoxy)ethyleneglycol-N,N,N',N'-tetraacetic acid,tetraacetoxymethyl ester:N,N,N',N'-Tetrakis(2-pyridylemethyl)ethylenediamine:1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-xantheny)]-2-(2-Amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid:1-[2-Amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-xantheny)]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid,pentaacetoxymethyl ester:1-[6-Amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid,pentapotassium salt:1-[6-Amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid,pentaacetoxymethyl ester:1-[6-Amino-5-(6-carboxy-2-indolyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid,pentapotassium salt:1-[6-Amino-5-(6-carboxy-2-indolyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid,pentaacetoxymethyl ester:8-Amino-2-[2-amino-5-methylphenoxy)methyl]-6-methoxyquinoline-N,N,N',N'-tetraacetic,tetrapotassium salt:8-Amino-2-[(2-amino-5-methylphenoxy)methyl]-6-methoxyquinoline-N,N,N',N'-tetraacetic,tetraacetoxymethyl ester:1-[(2-Amino-5-(3-dimethylamino-6-dimethylammonio-9-xanthenyl]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid,chloride:1-[(2-Amino-5-(3-dimethylamino-6-dimethylammonio-9-xanthenyl]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid,tetraacetoxymethyl ester,chloride:2'.7'-Bis(carboxyethyl)-4 or 5-carboxyfluoreescein:

【００６６】3'-O-Acetyl-2',7'-bis(carboxyethyl)-4 or 5-carboxyfluorescien,diacetoxymethyl ester:8-Amino-2-(trans-2-aminostyryl)-6-methoxyquinoline-N,N,N',N'-tetraacetic acid,tetrapotassium salt:8-Amino-2-(trans-2-aminostyryl)-6-methoxyquinoline-N,N,N',N'-tetraacetic acid,tetraacetoxymethyl ester:4,4'-Bis[｛6-[N,N-bis(2-hydroxyethyl)amino]-4-phenylamino-1,3,5-triazin-2-yl｝amino]-2,2'-stilbenedisulfonic acid,disodium salt:4.4'-Bis[｛6-[N,N-diethylamino]-4-phenylamino-1,3,5-triazin-2-yl｝amino]-2,2'-stilbenedisulfonic acid,disodium salt:4,4'-Bis[(6-methoxy-4-phenylamino-1,3,5-triazin-2-yl)amino]-2,2'-stilbenedisulfonic acid,disodium salt:1,3-Bis(1-pyrenyl)propane,1-(4-Trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatrieneiodide:N-[3-(1,5-Disulfonaphtyl)]-N'-[4-(2,2,6,6-tetramethylpiperidine-N-oxide)thiouurea,disodium salt:N-Ethoxycarbonylmethyl-6-methoxyquinolinium promide:6-Methoxy-N-(3-sulfopropyl)quinolinium,monohydrate:5-or-6-(N-Succinimidyloxycarbonyl)-3',6'-diacetylfluorescein:5-or-6-(N-Succiniumidyloxycarbonyl)-4',5'-diamethyl-3',6'-O,O'-diacetylfluorescein:5-or-6-(N-Succiniumidyloxycarbonyl)-4',5'-dichloro-3',6'-O,O'-diacetylfluorescein:

【００６７】3,6-bis-dimethylaminoacridine:9-aminoacridine:9-(4-diethylamino-methylbutylamino)-3-chloro-7-methoxyacridine:1-anilinonaphthalene-8-sulfonate:N-methyl-2-anilinonaphthalene-6-sulfonate:2-p-

toluidinyl-naphthalene-6-sulfonate:12(9-anthroyloxy)stearic acid:teramethyldiaminodiphenylketoimine hydrochloride:7-chloro-4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy:cyanine dyes:1,1'-dihexyl-2,2'-oxacarbocyanine:3,3'-dipropylthiadicarbocyanine:5-[(3-sulfopropyl-2(3H)-benzoxazolylidene)-2-butenylidene]-1,3-dibutyl-2-thiobarbituric acid:5-dimethylaminonaphthalene-1-sulfonamide:dansylamino-ethyltriphosphate:1-(5-dimethylaminonaphthalene-1-sulfonamide)-3-N,N,-dimethylaminopropane:1-(5-dimethylaminonaphthalene-1-sulfonamido)-propane-3-trimethlammonium:(N-dansyl)-aminoalkyl-β-D-galactopyranoside: ε-dansyl-L-lysine:dansylphosphaidyl-ethanolamine:1,6-diphenyl-1,3,5-hexatriene:2',4',5',7'-tetrabromofluorescein:1,N6-ethennoadenosine:2,7-diamino-9-phenylphenanthrium-10-ethyl-bromide:9-(o-carboxyphenyl)-6-hydroxy-3H-xanthen-3-one:

【００６８】7-amino-3-(β-D-ribrofuranosyl)pyrazolo(4,3-d)pyrimidine:4-benzoylamido-4'-aminostilbene-2-2'-disulfonate:1-acyl-2-[N-(4-nitrobenzo-2-oxa-1,3-diazolyl)aminocaproyl]phosphatridylcholine:β-naphthyltriphosphate:oxonol dye:bis[3-phenyl-5-oxoisoxazol-4-yl]pentamethineoxonol:bis[1,3-dibutylbarbituric-acid(5)]pentamethineoxonol: α(9,11,13,15-cis.trans,trans,cis)octadecatetraenoic acid:β(9,11,13,15-all,trans)octadecateraenoic acid:perylene:N-phenyl-1-napthylamine:pyrene:2,3-dimethyl-3,7-diamino-5-phenylphenazium:4-phenylspro[furan-2(3H),1'-futalan]-3,3'-dione:o-phthalic dicarboxaldehyde:1-dimethylaminonaphthalene-5-sulfonyl chloride:flurorescien isothiocyanate:7-chloro-4-nitrobenzo-2-oxal,3-diazole:N-dansyl aziridine:5-(iodoacetoamidoethyl)amino-naphthalene-1-sulfonate:5-iodoacetamido fluorescein:N-(1-anilinonaphthyl-4)maleimide:N-(7-dimethylamino-4-methylcoumarynul)maleimide:N-(3-pyrene)maleimide:eosin-5-iodoacetamide:fluorescein mercuty-acetate:2-[4'(2"-iodoacetamido)phenyl]aminonaphthalene-6-sulfonic acid:

【００６９】このうち、ｏ－フタルアルデヒド(o-phthalic dicarboxaldehyde) 等の多くの蛍光プローブは、ｐＨ６～１１．５の溶液中にあるとき安定した強い蛍光強度が保たれるので、安定した信号強度で画素を形成でき、良質な顕微鏡像が期待できる。よって、染色した試料をｐＨ６～１１．５の緩衝溶液中に浸して測定すれば、本発明の利点を十分活かした高画質の像が得られるであろう。

【００７０】第２実施例

図１０は本実施例の観察方法で用いられる走査型蛍光顕微鏡の構成を示す図である。ここに示す走査型蛍光顕微鏡は、第１実施例に示した顕微鏡の構成に加え、２つの偏光子４１，４２が夫々２倍波発振器１４と色素レーザ１３の射出側に配置されている。このように、偏光子４１，４２を備えることにより、２台の色素レーザーから発振されるレーザー光の偏光面を夫々独立して制御できるようになっている。そして、光の偏光面と試料２４を染色する分子の配光方向とは強い相関関係があることから、２つの波長の光の夫々の偏光面と前記分子の配向方向とが特定の角度をなすとき二重共鳴吸収過程が生じることを利用することにより、蛍光の消失の程度の変化から試料２４の空間的配向分布の情報が得られる。

【００７１】具体的には、２つの波長の光を試料２４上に同時に照射する際、２つの偏光子４１，４１を透過させることにより、夫々のレーザー光の偏光面を設定する。このとき、２つのレーザー光の偏光面は試料２４を染色する分子の空間的配向に対応しているので、特定の偏光条件が満たされたときのみ二重共鳴吸収を強く生じ、これに対応して特定の方向に配向した前記分子からの蛍光が消失する。この蛍光の減少量より、観察する試料２４の空間配向の状態の分析ができる。このように、本実施例による観察方法では、超解像性に加え、試料の組織の空間配向分析も行える。

【００７２】以上説明したように、本発明の顕微鏡の観察方法は特許請求の範囲に記載された特徴と併せ、以下（１）～（１０）に示すような特徴も備えている。

【００７３】（１）二重結合を有する分子を試料の染色のために用いることを特徴とする請求項１に記載の顕微鏡の観察方法。

【００７４】（２）化学基として少なくとも６員環を１つ含む分子を試料の染色のために用いることを特徴とする請求項１に記載の顕微鏡の観察方法。

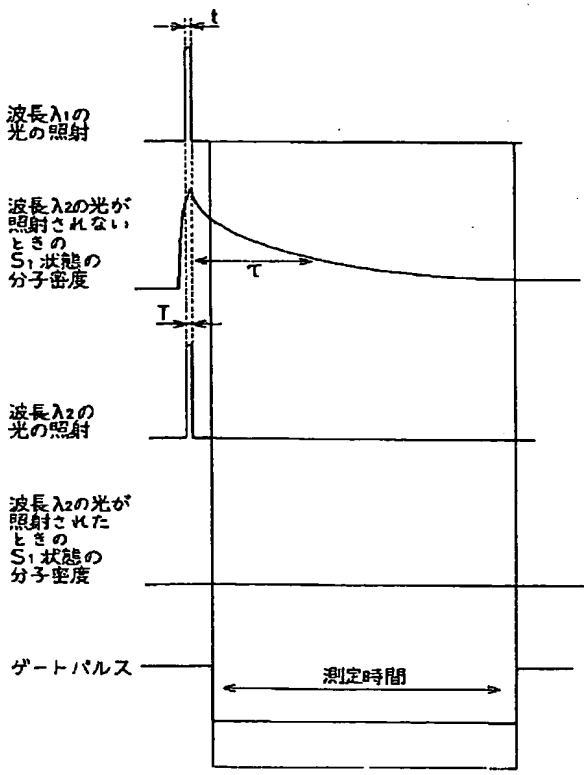
【００７５】（３）以下に示す分子の何れかを用いて試料の染色を行うようにしたことを特徴とする請求項１に記載の顕微鏡の観察方法。

4-Fluoro-7-sulfamoylbenzofurazan:3,6-Bis(dimethylamino)-10-dodecylacridinium bromide:4-Benzylamino-7-nitrobenzofurazan:4-Azidofluorescein diacetate,5,6-Dimethoxy-2-(4-hydrazinocarbonylphenyl)benzothiazole:3-Bromomethyl,6,7-dimethoxy-1-methyl-1,2-dihydroquinoxaline-2-one:4-Bromoethyl-7-methoxycoumarin:N-[4-(6-Dimethylamino-2-benzofurany]maleimide:1,2-Diamino-4,5-dimethoxybenzene,dihydrochloride:2,2'-Dihydroxy-6,6'-dinaphthyl disulfide:3-Chlorocarbonyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone:2-2'-Dithiobis(1-aminoaphthalene),Fluorescein-4-isothiocyanate:4-Amino-3-penten-2-one:4-(4-Methoxybenzylamino)-7-nitrobenzofurazan:N-[4-(5,6-Methylenedioxy-2-benzofuranyl)]maleimide:1,2-Diamino-4,5-methylenedioxybenzene:N-(9-Acridinyl)maleimide:4-Fluoro-7-nitrobenzofurazan,4-Chloro-7-nitrobenzofuraza

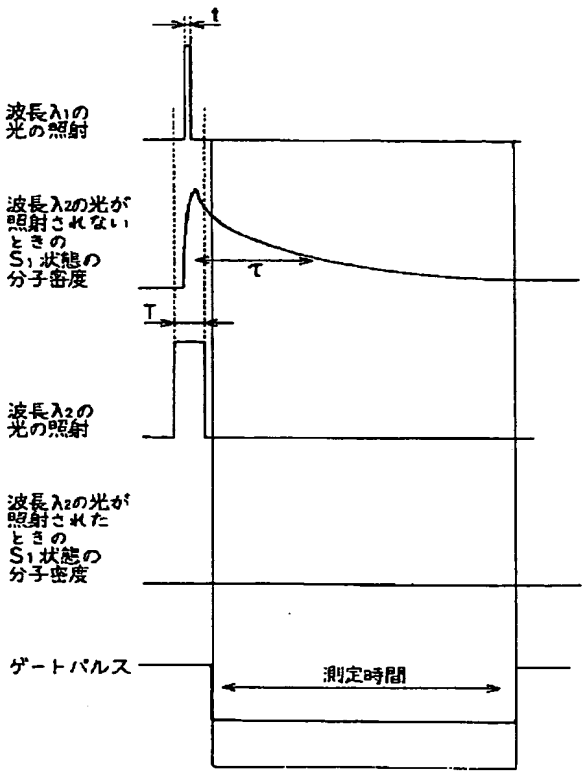
n:(S)-(-)-1-(2,3-Napthalenedicarboximidyl fluoride:2-(5-Chlorocarbonyl-2-oxazolyl)-5,6-methylenedioxybenzofuran:4-Chloro-7-sulfobenzofurazan,ammonium salt:4-Fluoro-7-sulfobenzofurazan,ammonium salt:Sulforthodamine 101 acid chloride:4-[4-(Dimethylamino)phenylazo]phenylisothiocyanate:3,5-Dinitrobenzoyl chloride:5-(4-Dimethylaminophenyl)-2,4-pentadienal:1,3-Diphenyl-2-thiobarbituric acid:O-(4-Nitrobenzyl)-N,N'-diisopropylisourea:O-(4-Nitrobenzyl)hydroxylamine,hydrochloride:N,N,N'-Triethyl-N'-[N-(N-succinimidyloxycarbonyl)pentyl]-9-cyanopyronine chloride:N,N,N'-Triethyl-N'- {5-[N''-(2-maleimidoethyl)piperazinocarbonyl]pentyl } 9-cyanopyronine chloride:2,4'-Dibromoacetophenone:N-Succinimidyl-4-nitrophenylacetate:O,O'-Bis(2-aminophenyl)ethyleneglycol-N,N,N',N'-tetraacetic acid,tetraapotassium,hydrate:O,O'-Bis(2-aminophenyl)ethyleneglycol-N,N,N',N'-tetraacetic acid,tetraacetoxymethyl ester:O,O'-Bis(2-amino-5-fluorophenoxy)ethyleneglycol-N,N,N',N'-tetraacetic acid,tetraapotassium,hydrate:O,O'-Bis(2-amino-5-fluorophenoxy)ethyleneglycol-N,N,N',N'-tetraacetic acid,tetraacetoxymethyl ester:N,N,N',N'-Tetrakis(2-pyridylemethyl)ethylenediamine:1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-xantheny)]-2-(2-Amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid:1-[2-Amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-xantheny)]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid,pentaacetoxymethyl estar:1-[6-Amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid,pentapotassium salt:1-[6-Amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid,pentaacetoxymethyl ester:1-[6-Amino-5-(6-carboxy-2-indolyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid,pentapotassium salt:1-[6-Amino-5-(6-carboxy-2-indolyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid,pentaacetoxymethyl ester:8-Amino-2-[2-amino-5-methylphenoxy)methyl]-6-methoxyquinoline-N,N,N',N'-tetraacetic,tetrapotassium salt:8-Amino-2-[(2-amino-5-methylphenoxy)methyl]-6-methoxyquinoline-N,N,N',N'-tetraacetic,tetraacetoxymethyl ester:1-[(2-Amino-5-(3-dimethylamino-6-dimethylammonio-9-xanthenyl]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid,chloride:1-[(2-Amino-5-(3-dimethylamino-6-dimethylammonio-9-xanthenyl]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid,tetraacetoxymethyl ester,chloride:2'.7'-Bis(carboxyethyl)-4 or 5-carboxyfluoreescein:3'-O-Acetyl-2',7'-bis(carboxyethyl)-4 or 5-carboxyfluorescien,diacetoxymethyl ester:8-Amino-2-(trans-2-aminostyryl)-6-methoxyquinoline-N,N,N',N'-tetraacetic acid,tetrapotassium salt:8-Amino-2-(trans-2-aminostyryl)-6-methoxyquinoline-N,N,N',N'-tetraacetic acid,tetraacetoxymethyl ester:4,4'-Bis[{6-[N,N-bis(2-hydroxyethyl)amino]-4-phenylamino-1,3,5-triazin-2-yl } amino]-2,2'-stilbenedisulfonic acid,disodium salt:4.4'-Bis[{6-[N,N-diethylamino]-4-phenylamino-1,3,5-triazin-2-yl } amino]-2,2'-stilbenedisulfonic acid,disodium salt:4,4'-Bis[(6-methoxy-4-phenylamino-1,3,5-triazin-2-yl)amino]-2,2'-stilbenedisulfonic acid,disodium salt:1,3-Bis(1-pyrenyl)propane,1-(4-Trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatrieneiodide:N-[3-(1,5-Disulfonaphtyl)]-N'-[4-(2,2,6,6-tetramethylpiperidine-N-oxide)thiouurea,disodium salt:N-Ethoxycarbonylmethyl-6-methoxyquinolinium promide:6-Methoxy-N-(3-sulfopropyl)quinolinium,monohydrate:5-or-6-(N-Succinimidyloxycarbonyl)-3',6'-diacetylfluorescein:5-or-6-(N-Succiniumidyloxycarbonyl)-4',5'-diamethyl-3',6'-O,O'-diacetylfluorescein:5-or-6-(N-Succiniumidyloxycarbonyl)-4',5'-dichloro-3',6'-O,O'-diacetylfluorescein:3,6-bis-dimethylaminoacridine:9-aminoacridine:9-(4-diethylamino-methylbutylamino)-3-chloro-7-methoxyacridine:1-anilinonaphthalene-8-sulfonate:N-methyl-2-anilinonaphthalene-6-sulfonate:2-p-toluidinyl-naphthalene-6-sulfonate:12(9-anthroyloxy)stearic acid:teramethyldiaminodiphenylketoimine hydrochloride:7-chloro-4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy:cyanine dyes:1,1'-dihexyl-2,2'-oxacarbocyanine:3,3'-dipropylthiadicarbocyanine:5-[(3-sulfopropyl-2(3H)-benzoxazolyliden e)-2-butenylidene]-1,3-dibutyl-2-thiobarbituric acid:5-dimethylaminonaphthalene-1-sulfonamide:dansylamino-ethyltriphosphate:1-(5-dimethylaminonaphthalene-1-sulfonamide)-3-N,N,-dimethyaminopropane:1-(5-dimethylaminonaphthalene-1-sulfonamido)-propane-3-trimethlammonium:(N-dansyl)-aminoalkyl- β-D-galactopyranoside: ε-dansyl-L-lysine:dansylphosphaidyl-ethanolamine:1,6-diphenyl-1,3,5-hexatriene:2',4',5',7'-tetrabromofluorescein:1,N6-ethennoadenosine:2,7-diamino-9-phenylphenanthrium-10-ethyl-bromide:9-(o-carboxyphenyl)-6-hydroxy-3H-xanthen-3-one:7-amino-3-(β-D-ribrofuranosyl)pyrazolo(4,3-d)pyrimidine:4-benzoylamido-4'-aminostilbene-2-2'-disulfonate:1-acyl-2-[N-(4-nitrobenzo-2-oxa-1,3-diazolyl)aminocaproyl]phosphatridylcholine:β-naphthyltriphosphate:oxonol dye:bis[3-phenyl-5-oxoisoxazol-4-yl]pentamethineoxonol:bis[1,3-dibutylbarbituric-ac

n:(S)-(-)-1-(2,3-Napthalenedicarboximidyl fluoride:2-(5-Chlorocarbonyl-2-oxazolyl)-5,6-methylenedioxybenzofuran:4-Chloro-7-sulfobenzofurazan,ammonium salt:4-Fluoro-7-sulfobenzofurazan,ammonium salt:Sulforthodamine 101 acid chloride:4-[4-(Dimethylamino)phenylazo]phenylisothiocyanate:3,5-Dinitrobenzoyl chloride:5-(4-Dimethylaminophenyl)-2,4-pentadienal:1,3-Diphenyl-2-thiobarbituric acid:O-(4-Nitrobenzyl)-N,N'-diisopropylisourea:O-(4-Nitrobenzyl)hydroxylamine,hydrochloride:N,N,N'-Triethyl-N'-[N-(N-succinimidyloxycarbonyl)pentyl]-9-cyanopyronine chloride:N,N,N'-Triethyl-N'- {5-[N"-(2-maleimidoethyl)piperazinocarbonyl]pentyl } 9-cyanopyronine chloride:2,4'-Dibromoacetophenone:N-Succinimidyl-4-nitrophenylacetate:O,O'-Bis(2-aminophenyl)ethyleneglycol-N,N,N',N'-tetraacetic acid,tetraapotassium,hydrate:O,O'-Bis(2-aminophenyl)ethyleneglycol-N,N,N',N'-tetraacetic acid,tetraacetoxymethyl ester:O,O'-Bis(2-amino-5-fluorophenoxy)ethyleneglycol-N,N,N',N'-tetraacetic acid,tetraapotassium,hydrate:O,O'-Bis(2-amino-5-fluorophenoxy)ethyleneglycol-N,N,N',N'-tetraacetic acid,tetraacetoxymethyl ester:N,N,N',N'-Tetrakis(2-pyridylemethyl)ethylenediamine:1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-xantheny)]-2-(2-Amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid:1-[2-Amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-xantheny)]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid,pentaacetoxymethyl estar:1-[6-Amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid,pentapotassium salt:1-[6-Amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid,pentaacetoxymethyl ester:1-[6-Amino-5-(6-carboxy-2-indolyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid,pentapotassium salt:1-[6-Amino-5-(6-carboxy-2-indolyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid,pentaacetoxymethyl ester:8-Amino-2-[2-amino-5-methylphenoxy)methyl]-6-methoxyquinoline-N,N,N',N'-tetraacetic,tetrapotassium salt:8-Amino-2-[(2-amino-5-methylphenoxy)methyl]-6-methoxyquinoline-N,N,N',N'-tetraacetic,tetraacetoxymethyl ester:1-[(2-Amino-5-(3-dimethylamino-6-dimethylammonio-9-xanthenyl]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid,chloride:1-[(2-Amino-5-(3-dimethylamino-6-dimethylammonio-9-xanthenyl]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid,tetraacetoxymethyl ester,chloride:2'.7'-Bis(carboxyethyl)-4 or 5-carboxyfluoreescein:3'-O-Acetyl-2',7'-bis(carboxyethyl)-4 or 5-carboxyfluorescien,diacetoxymethyl ester:8-Amino-2-(trans-2-aminostyryl)-6-methoxyquinoline-N,N,N',N'-tetraacetic acid,tetrapotassium salt:8-Amino-2-(trans-2-aminostyryl)-6-methoxyquinoline-N,N,N',N'-tetraacetic acid,tetraacetoxymethyl ester:4,4'-Bis[{6-[N,N-bis(2-hydroxyethyl)amino]-4-phenylamino-1,3,5-triazin-2-yl } amino]-2,2'-stilbenedisulfonic acid,disodium salt:4.4'-Bis[{6-[N,N-diethylamino]-4-phenylamino-1,3,5-triazin-2-yl } amino]-2,2'-stilbenedisulfonic acid,disodium salt:4,4'-Bis[(6-methoxy-4-phenylamino-1,3,5-triazin-2-yl)amino]-2,2'-stilbenedisulfonic acid,disodium salt:1,3-Bis(1-pyrenyl)propane,1-(4-Trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatrieneiodide:N-[3-(1,5-Disulfonaphtyl)]-N'-[4-(2,2,6,6-tetramethylpiperidine-N-oxide)thiouurea,disodium salt:N-Ethoxycarbonylmethyl-6-methoxyquinolinium promide:6-Methoxy-N-(3-sulfopropyl)quinolinium,monohydrate:5-or-6-(N-Succinimidyloxycarbonyl)-3',6'-diacetylfluorescein:5-or-6-(N-Succiniumidyloxycarbonyl)-4',5'-diamethyl-3',6'-O,O'-diacetylfluorescein:5-or-6-(N-Succiniumidyloxycarbonyl)-4',5'-dichloro-3',6'-O,O'-diacetylfluorescein:3,6-bis-dimethylaminoacridine:9-aminoacridine:9-(4-diethylamino-methylbutylamino)-3-chloro-7-methoxyacridine:1-anilinonaphthalene-8-sulfonate:N-methyl-2-anilinonaphthalene-6-sulfonate:2-p-toluidinyl-naphthalene-6-sulfonate:12(9-anthroyloxy)stearic acid:teramethyldiaminodiphenylketoimine hydrochloride:7-chloro-4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy:cyanine dyes:1,1'-dihexyl-2,2'-oxacarbocyanine:3,3'-dipropylthiadicarbocyanine:5-[(3-sulfopropyl-2(3H)-benzoxazolyliden e)-2-butenylidene]-1,3-dibutyl-2-thiobarbituric acid:5-dimethylaminonaphthalene-1-sulfonamide:dansylamino-ethyltriphosphate:1-(5-dimethylaminonaphthalene-1-sulfonamide)-3-N,N,-dimethyaminopropane:1-(5-dimethylaminonaphthalene-1-sulfonamido)-propane-3-trimethlammonium:(N-dansyl)-aminoalkyl- β-D-galactopyranoside: ε-dansyl-L-lysine:dansylphosphaidyl-ethanolamine:1,6-diphenyl-1,3,5-hexatriene:2',4',5',7'-tetrabromofluorescein:1,N6-ethennoadenosine:2,7-diamino-9-phenylphenanthrium-10-ethyl-bromide:9-(o-carboxyphenyl)-6-hydroxy-3H-xanthen-3-one:7-amino-3-(β-D-ribrofuranosyl)pyrazolo(4,3-d)pyrimidine:4-benzoylamido-4'-aminostilbene-2-2'-disulfonate:1-acyl-2-[N-(4-nitrobenzo-2-oxa-1,3-diazolyl)aminocaproyl]phosphatridylcholine:β-naphthyltriphosphate:oxonol dye:bis[3-phenyl-5-oxoisoxazol-4-yl]pentamethineoxonol:bis[1,3-dibutylbarbituric-ac

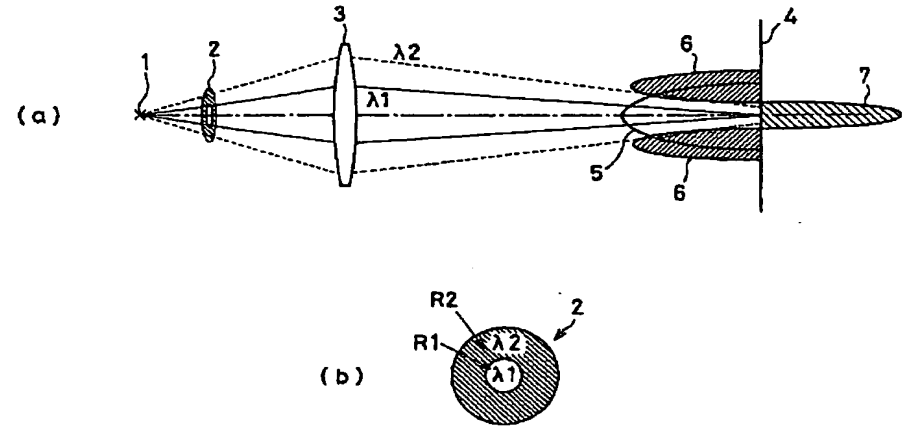
【図３】


t
波長λ1の光の照射
波長λ2の光が照射されないときのS1状態の分子密度
τ
T
波長λ2の光の照射
波長λ2の光が照射されたときのS1状態の分子密度
ゲートパルス
測定時間

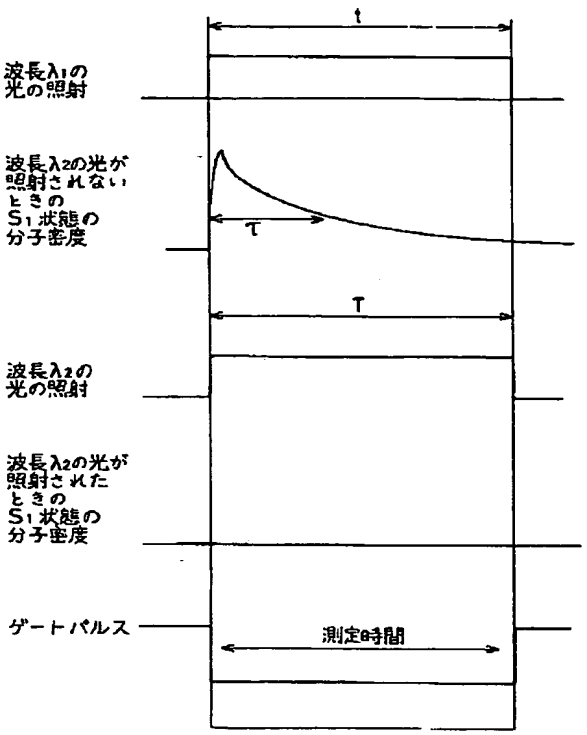
【図４】


t
波長λ1の光の照射
波長λ2の光が照射されないときのS1状態の分子密度
τ
T
波長λ2の光の照射
波長λ2の光が照射されたときのS1状態の分子密度
ゲートパルス
測定時間

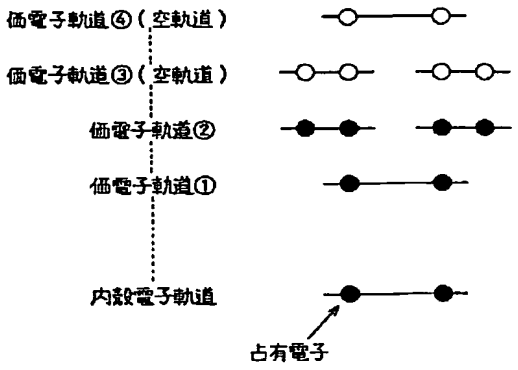
【図６】


(a)
1
2
3
λ2
λ1
4
6
7
5
6
(b)
R2
R1
2
λ2
λ1

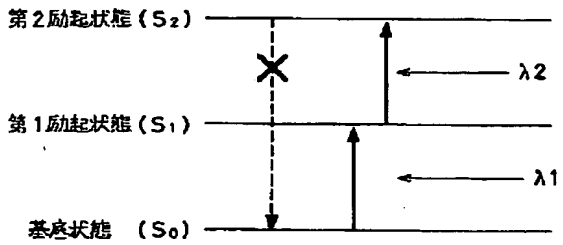
【図５】


t
波長λ1の光の照射
波長λ2の光が照射されないときのS1状態の分子密度
τ
T
波長λ2の光の照射
波長λ2の光が照射されたときのS1状態の分子密度
ゲートパルス
測定時間

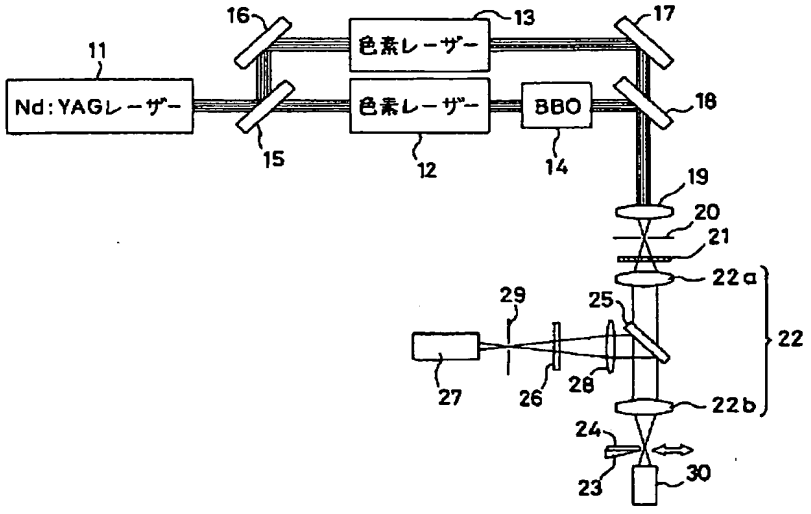
【図１１】


価電子軌道④（空軌道）
価電子軌道③（空軌道）
価電子軌道②
価電子軌道①
内殻電子軌道
占有電子

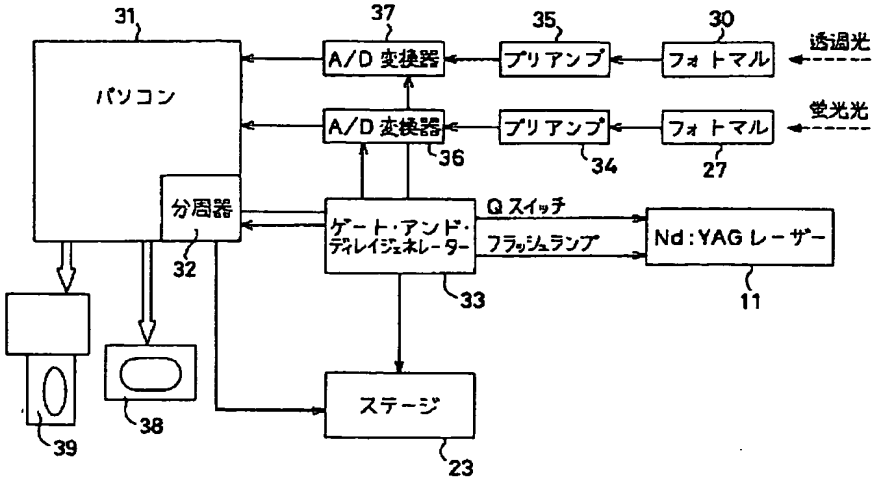
【図１５】


第２励起状態（S2）
第１励起状態（S1）
基底状態　（S0）
λ2
λ1

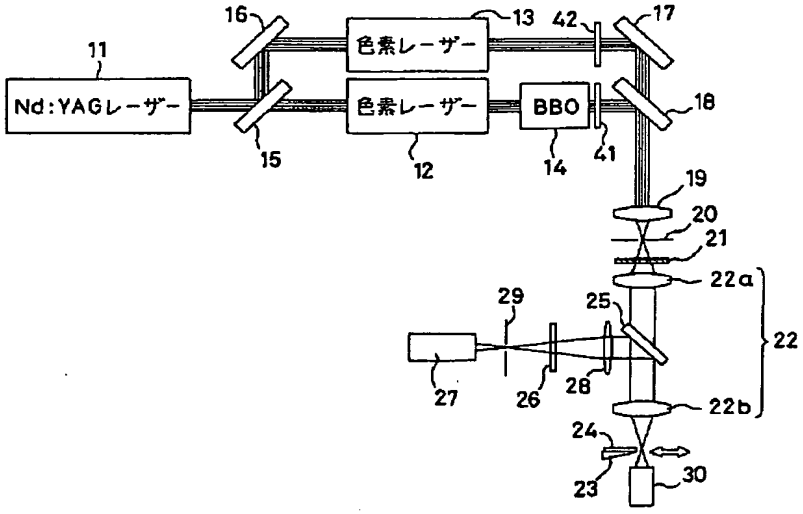
【図８】


11
Nd:YAGレーザー
16
15
色素レーザー
13
色素レーザー
12
17
BBO
14
18
19
20
21
22a
22
22b
29
25
27
26
28
24
23
30

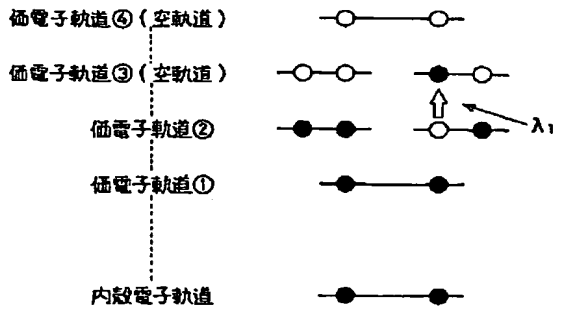
【図９】



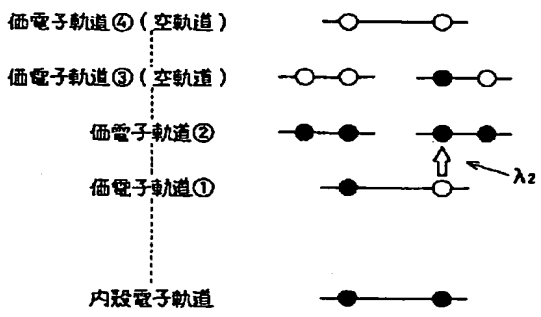
【図１０】



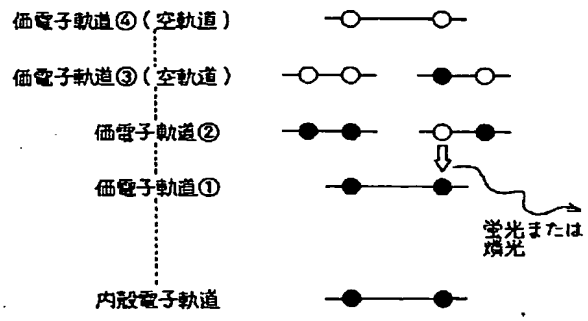
【図１２】



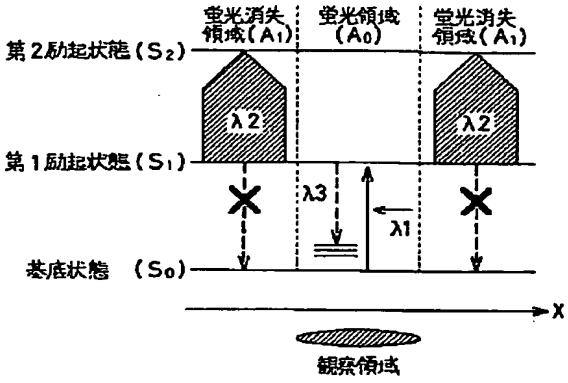
【図１３】



【図１４】


価電子軌道④（空軌道）
価電子軌道③（空軌道）
価電子軌道②
価電子軌道①
蛍光または燐光
内殻電子軌道

【図１６】


蛍光消失領域（A1）
蛍光領域（A0）
蛍光消失領域（A1）
第２励起状態（S2）
第１励起状態（S1）
基底状態　（S0）
λ2
λ3
λ1
X
観察領域

PATENT ABSTRACTS OF JAPAN

(11)Publication number : 11-095120
(43)Date of publication of application : 09.04.1999

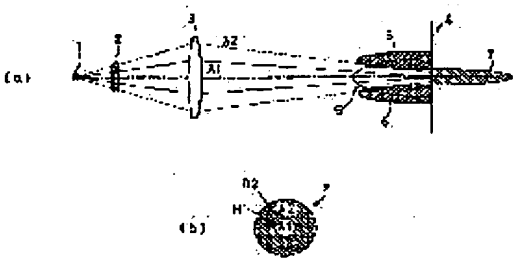
(51)Int.Cl. G02B 21/16

(21)Application number : 09-255444
(22)Date of filing : 19.09.1997

(71)Applicant : OLYMPUS OPTICAL CO LTD
(72)Inventor : KASHIMA SHINGO
IKETAKI YOSHINORI

(54) OBSERVING METHOD OF MICROSCOPE

(57)Abstract:
PROBLEM TO BE SOLVED: To provide an observing method for obtaining a microscopic image of high spacial resolving power by using a molecule suited to the principle of a double resonance absorption process for dyeing a sample as an object to be observed and irradiating the dyed sample with light having plural wavelengths with good timing in the observation of a fluorescent microscope.
SOLUTION: In this observing method, light having a wavelength λ1 is converged on the surface 4 of a sample and the surface 4 of the sample is irradiated with light having a wavelength λ2 in a defocused state through an aperture 2 having a ring structure and an optical system 3 provided with a different converging position in the region of wavelengths λ1, λ2 from among light beams having wavelengths λ1, λ2 emitted from a light source 1. Consequently, the spacial resolving power of the microscope is improved beyond the condition of diffraction limit by the wavelength.



LEGAL STATUS

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* NOTICES *

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CLAIMS

[Claim(s)]
[Claim 1] Wavelength lambda 1 which excites this molecule to the 1st excitation state using the molecule which has three quantum states including a ground state at least for dyeing of a sample The light source which emits light, Wavelength lambda 2 which excites the molecule of the 1st excitation state to the 2nd excitation state or an excitation state higher than this The light source which emits light, Said wavelength lambda 1 Light and wavelength lambda 2 Condensing optical system which makes light condense on said sample, The luminescence detector which detects luminescence at the time of the molecule which said excited sample was dyed carrying out deexcitation to a ground state, Said wavelength lambda 1 The exposure field and said wavelength lambda 2 of light It has a heavy means to pile up a part of exposure field of light, it lets this heavy means pass, and is said wavelength lambda 1. Light and wavelength lambda 2 By irradiating said sample, light In the optical microscope which controlled the field of luminescence at the time of carrying out deexcitation from the 1st excitation state to a ground state The observation approach of the microscope characterized by using a molecule with a thermal-relaxation process more dominant than the relaxation process according [the transition when carrying out deexcitation to a ground state] to fluorescence as a fluorescent probe molecule from the high-order energy state except the 1st excitation state as a molecule which said sample is dyed.
[Claim 2] It is said wavelength lambda 1 independently or subordinately. Light and wavelength lambda 2 The observation approach of the microscope according to claim 1 which enabled it to carry out adjustable [of the polarization condition of light].
[Claim 3] Said wavelength lambda 1 to said sample Light and wavelength lambda 2 The observation approach of the microscope according to claim 1 or 2 which set irradiation time of light to 1/10 of the life time of fluorescence of a molecule which dyes this sample.

[Translation done.]

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DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention] This invention relates to the observation approach which used the optical microscope. It is illuminating the dyed sample by the light of two or more wavelength in detail, and is related with the observation approach using the scanning fluorescence microscope which made it possible to acquire good image quality with high spatial resolving power.
[0002]
[Description of the Prior Art] The history of an optical microscope is old and the microscope various type has been developed to current. Moreover, the still more highly efficient microscope system is developed by advance of circumference techniques including a laser technique in recent years and electronic imaging technology. The highly efficient microscope which used the double resonance absorption process emitted by illuminating a sample with the light of two or more wavelength by JP,8-248200,A etc. in such a background, and made possible not only control of the contrast of the image obtained but the chemical analysis is proposed. the following and this microscope -- a spectrum -- it explains from a position [-like].
[0003] Drawing 11 is drawing showing the electronic structure of the valence-electron orbit of the molecule which constitutes a sample. Under the microscope currently indicated by said JP,8-248200,A, a double resonance absorption process can be used, a specific molecule can be chosen, and the absorption and the fluorescence resulting from specific optical transition can be observed. Drawing 12 thru/or 14 use and explain this principle further.
[0004] First, the electron of the valence-electron orbit which the molecule of the ground state (the following and S0 a condition is called) shown in drawing 11 has is excited by the light of a certain wavelength (lambda 1), as shown in drawing 12 R> 2 (the 1st excitation state: the following and S1 a condition is called). Next, as similarly shown in drawing 13 , it excites by the light of another wavelength (lambda 2) (below : called the 2nd excitation state and S2 a condition is called). This S2 The molecule which changed into the condition emits light in fluorescence or phosphorescence, as shown in drawing 14 after this, and it is S0. It returns to a condition. The observation approach under the microscope using a double resonance absorption process observes an absorption image and a luminescence image using luminescence of the fluorescence and phosphorescence which were shown in drawing 12 , the absorption process shown in 13, or drawing 14 .
[0005] With this observation approach, they are the resonance wavelengths lambda 1, such as laser light, first. S1 as shown in drawing 12 in the molecule which constitutes a sample using light It is S1 although a condition is excited. The molecularity in the unit volume in a condition increases as the luminous intensity to irradiate increases. Moreover, a linear absorption coefficient is given by the product of the absorption cross section per molecule, and the molecularity per unit volume. Therefore, wavelength lambda 1 first irradiated in the excitation state as shown in drawing 13 Resonance wavelength lambda 2 irradiated following light The receiving linear absorption coefficient is said wavelength lambda 1. It is dependent on luminous intensity. Namely, wavelength lambda 2 The linear absorption coefficient to light is wavelength lambda 1. It can control by luminous intensity. This is wavelength lambda 1. Wavelength lambda 2 A sample is irradiated with two waves of light, and it is wavelength lambda 2. If the transmission image by light is photoed, the contrast of a transmission image will be wavelength lambda 1. It is shown that it is completely controllable by the quantity of light.
[0006] On the other hand, it is S2 as shown in drawing 14 . When the deexcitation condition by the fluorescence or phosphorescence from a condition is possible, the luminescence reinforcement is S1. It is proportional to the number of the molecules in a condition. Therefore, also when using as a fluorescence

microscope, it becomes controllable [image contrast]. Moreover, this approach makes possible not only control of contrast but the chemical analysis.
[0007] Here, the outermost shell valence-electron orbit shown in drawing 11 has an energy level peculiar to each molecule. Therefore, wavelength lambda 1 The fluorescence at the time of irradiating light changes with molecules. To coincidence, it is wavelength lambda 2. Also when light is irradiated, the fluorescence of each molecule proper is emitted. Like before, even if it is the case where irradiate a sample and the light of single wavelength is observed, it is possible to observe to some extent specific absorption image or fluorescence image of a molecule. However, since the wavelength field of the absorption band of some molecules generally overlaps, exact identification of the chemical composition of a sample is impossible.
[0008] On the other hand, with the observation approach using a double resonance absorption process, it is wavelength lambda 1. And wavelength lambda 2 Since the molecule which absorbs or emits light by two waves of light is limited, identification of the chemical composition of a sample more exact than the conventional approach is attained. Moreover, since only the light which has a specific electric field vector to a molecule shaft is strongly absorbed when exciting the valence electron, it is wavelength lambda 1. And wavelength lambda 2 If the polarization direction is defined and absorption or a fluorescence image is photoed, the same molecule will also become possible to identification of the direction of orientation.
[0009] Furthermore, recently, the fluorescence microscope with the high spatial resolving power exceeding the analysis limitation using a double resonance absorption process is proposed. The principle of this microscope is explained using drawing 15 . Drawing 15 is the conceptual diagram showing the double resonance absorption process in a certain molecule. S0 The molecule in a condition is wavelength lambda 1. It is S1 at light. It is excited by the condition and is wavelength lambda 2 further. It is S2 at light. Signs that it is excited by the condition are shown. And S2 of this molecule It is shown that the fluorescence in a condition is very weak.
[0010] By the way, a very interesting phenomenon is expectable to the molecule which has such optical property. This is explained using drawing 16 . Like drawing 15 , although drawing 16 is the conceptual diagram of a double resonance absorption process, it prepares the X-axis in an axis of abscissa, and is expressing the flare of air clearance. And wavelength lambda 2 Space field A1 which can irradiate light Wavelength lambda 2 Space field A0 where light is not irradiated It is shown.
[0011] Space field A0 It is wavelength lambda 1 then. It is S1 by excitation by light. Many molecules of a condition are generated. this time -- space field A0 from -- wavelength lambda 3 Luminescence of fluorescence is seen. However, space field A1 It is wavelength lambda 2 then. Since light was irradiated, it is S1. S2 of almost immediate more a high order [molecule / of a condition] It is excited by the condition and is S1. The molecule of a condition stops existing. This phenomenon is checked with some molecules. this phenomenon -- wavelength lambda 3 fluorescence -- perfect -- being lost -- moreover -- S2 since the fluorescence from a condition does not exist from the first -- space field A1 **** -- the fluorescence itself is controlled completely. Therefore, it is A0 that fluorescence exists in a space field. It turns out that it is only a field.
[0012] Such a result has very important semantics in the microscope field. That is, in the conventional scanning laser microscope, laser light is condensed, a microbeam is formed and an observation sample top is scanned. At this time, the exposure field of a microbeam becomes settled to the diffraction limitation which becomes settled on the numerical aperture and wavelength of a condenser lens, and the spatial resolving power beyond it cannot be expected theoretically. However, as shown in drawing 16 , it is wavelength lambda 1 and lambda 2. By doubling two kinds of wavelength in skillful **** spatially, it is wavelength lambda 2 as mentioned above. A fluorescence field is controlled by the exposure of light. This time lambda 1, for example, wavelength, When its attention is paid to the exposure field of light, a fluorescence field is the wavelength lambda 1 which becomes settled on the numerical aperture and wavelength of a condenser lens. It is narrower than an exposure field and improvement in spatial resolving power is measured substantially. Therefore, if this principle is used, the fluorescence microscope exceeding a diffraction limitation is realizable (super resolution microscope using a double resonance absorption process).
[0013]
[Problem(s) to be Solved by the Invention] By the way, in a fluorescence microscope, the special molecule called a fluorescence labeler usually dyes a sample (mainly living body cell), and observation is performed. This fluorescence labeler will emit comparatively strong fluorescence, if the light of specific wavelength is absorbed. then -- as the example actually applied to observation with a fluorescence microscope although it was thought that it was very effective when using this fluorescence labeler for the above-mentioned observation approach -- a tryptophan and an adenine -- including, that effectiveness is shown very much

only about some molecules -- it is not alike too much. In addition, this tryptophan and adenine are the amino acid molecule or nucleobase included in the living body cell, and are a special molecule which is in intracellular and emits private fluorescence.
[0014] As for the molecule observable by no dyeing, the class is actually restricted, and many kinds of molecules cannot observe the presentation in no dyeing. A fluorescence labeler has the outstanding property that only the specific molecule for the purpose of observation can be dyed by choosing the functional group. Wavelength lambda 1 which selects the fluorescence labeler molecule suitable for the above-mentioned principle, and is moreover used in order are adapted for the super resolution microscope using a double resonance absorption process and to fully utilize the function And wavelength lambda 2 It is necessary to examine the exposure timing to the sample of two light. However, there are not suitable timing which irradiates light, and an example the fluorescence labeler molecule was concretely indicated to be until now.
[0015] Then, in view of the trouble of this conventional technique, this invention aims at offering the observation approach of obtaining the microscope image of high spatial resolving power in the dyed sample by irradiating the light of two or more wavelength to good timing in observation of a fluorescence microscope using the molecule which suits dyeing of the sample which is the observation specified substance at the principle of a double resonance absorption process.
[0016]
[Means for Solving the Problem] In order to attain the above-mentioned purpose, the observation approach of the microscope by this invention Wavelength lambda 1 which excites this molecule to the 1st excitation state using the molecule which has three quantum states including a ground state at least for dyeing of a sample The light source which emits light, Wavelength lambda 2 which excites the molecule of the 1st excitation state to the 2nd excitation state or an excitation state higher than this The light source which emits light, Said wavelength lambda 1 Light and wavelength lambda 2 Condensing optical system which makes light condense on said sample, The luminescence detector which detects luminescence at the time of the molecule which said excited sample was dyed carrying out deexcitation to a ground state, Said wavelength lambda 1 The exposure field and said wavelength lambda 2 of light It has a heavy means to pile up a part of exposure field of light, it lets said heavy means pass, and is said wavelength lambda 1. Light and wavelength lambda 2 By irradiating said sample, light In the optical microscope which controlled the field of luminescence at the time of carrying out deexcitation from the 1st excitation state to a ground state As a molecule which said sample is dyed, it is characterized by using a molecule with a thermal-relaxation process more dominant than the relaxation process according [the transition when carrying out deexcitation to a ground state] to fluorescence as a fluorescent probe molecule from the high-order energy state except the 1st excitation state.
[0017] Furthermore, observation of the microscope of this invention is said wavelength lambda 1 independently or subordinately. Light and wavelength lambda 2 It is characterized by enabling it to carry out adjustable [of the polarization condition of light]. Moreover, said wavelength lambda 1 to said sample Light and wavelength lambda 2 It is characterized also by setting irradiation time of light to 1/10 of the life time of fluorescence of a molecule which dyes said sample.
[0018]
[Embodiment of the Invention] In this invention, it has three quantum states including a ground state at least, and is based on dyeing a sample from the high-order energy state except the 1st excitation state using a molecule with a dominant thermal-relaxation process rather than the relaxation process according [the transition when carrying out deexcitation] to fluorescence to a ground state. And observation under the super resolution microscope using a double resonance absorption process is enabled by using this kind of molecule. To the biological material which gave the biochemical dyeing technique using this molecule, it is wavelength lambda 1. It is S0 by irradiating light. A condition to S1 A condition is excited and followed and it is wavelength lambda 2 immediately. By exciting to high order quantum level by light, it is S1. The fluorescence emitted in the condition is controlled. This invention uses the optical property of such a molecule, controls a spatial fluorescence field artificially, and aims at improvement in spatial resolving power. Hereafter, the optical property of this molecule is explained from a quantum chemistry-position.
[0019] Generally, each atom which constitutes a molecule is tied by sigma or pi bonding. for example, Sankyo Publishing -- according to "a guide (1991) to a quantum chemistry ingredient (Hirohiko Adachi)" -- the molecular orbital of a molecule -- sigma molecular orbital or pi molecular orbital -- **** -- it gets down and the electron which exists in these molecular orbitals is bearing the important role which combines each atom. Especially, the electron of sigma molecular orbital combined each atom strongly, and has determined the interatomic distance of the intramolecular which is the frame of a molecule.

[0020] On the other hand, it hardly contributes to each atomic union, but the electron of pi molecular orbital is rather bound to the whole molecule by the very weak force. If the electron in sigma molecular orbital is excited with light in many cases, atomic spacing of a molecule will change a lot and the big structural change including dissociation of a molecule will arise. Consequently, atomic kinetic energy and in order to change structurally, most energy which light gave to the molecule changes a form into heat energy. Therefore, excitation energy is not consumed with the gestalt of a light called fluorescence. Moreover, since a structural change of a molecule occurs in a high speed extremely (time amount shorter than Pico sec), even if fluorescence arises in the process, it will become what has the very short life time of fluorescence.
[0021] However, even if it excites the electron of pi molecular orbital to this, the structure of a molecule itself hardly changes but it has the property which emits and carries out deexcitation of the fluorescence to high-order quantum dissociation level to a long duration stop and the order of nsec. According to the quantum chemistry, it is equivalent that a molecule has pi molecular orbital and to have a double bond, and it becomes a requirement to select the molecule which has a double bond in abundance in the fluorescence labeler molecule used by this invention.
[0022] Furthermore, a molecule with a double bond is also set in 6 membered-ring molecules, such as benzene and pyrazine, and it is S2. It is known that the fluorescence from a condition is very weak (M. Fujii et.al.Chem.Phys.Lett.171 (1990) 341). Therefore, it will be S1 if the molecule containing 6 membered-ring molecules, such as benzene and pyrazine, is selected as a fluorescence labeler. It is long and, moreover, the life of the fluorescence from a condition is S1 by optical pumping. A condition to S2 Since the fluorescence produced from a molecule can be easily controlled by exciting to a condition, the approach of this invention can be performed effectively. That is, if it observes by these fluorescence labeler molecules dyeing, since the fluorescence image of a sample is not only observable with resolution between altitude, but only the specific chemistry organization of a biological material can be alternatively dyed by adjusting the chemical group of the side chain of the molecule, it can analyze to the chemical composition of a detailed sample.
[0023] Generally, since a double resonance absorption process occurs when wavelength, a polarization condition, etc. of two light fulfill specific conditions, it can know the structure of a very detailed molecule by using this. That is, there is a strong correlation in the direction of orientation of the plane of polarization of light, and a molecule, and when the plane of polarization of each light of two wavelength and the direction of orientation of a molecule make a specific include angle, a double resonance absorption process occurs strongly. Therefore, since extent of disappearance of fluorescence changes by turning the light of two wavelength to a sample side, irradiating coincidence, and rotating the plane of polarization of each light, if this situation is observed, the information on the space orientation of the organization of a sample will also be acquired. Furthermore, adjusting the wavelength of two light to irradiate, without rotating this plane of polarization can also know the space orientation of the organization of a sample.
[0024] As mentioned above, according to the approach of this invention, it turns out that the analysis capacity for it to be high besides super resolution nature is acquired.
[0025] Furthermore, by this invention, the approach of irradiating a sample also proposes light as selection of the fluorescence labeler molecule which can generate fluorescence control effectively to proper timing in the observation approach using the super resolution microscope using a double resonance absorption process. This principle is explained using drawing 1 .
[0026] The observational method under the super resolution microscope using a double resonance absorption process is wavelength lambda 1. A fluorescence labeler molecule is excited by light and it is wavelength lambda 2. It is based on erasing the fluorescence with light. Drawing 1 is lambda 1 and lambda 2. The timing which irradiates the light of two kinds of wavelength at a sample is shown. According to this drawing, it turns to a sample, and only time amount t is wavelength lambda 1 first. Light is irradiated and it is wavelength lambda 2 succeedingly. The time amount which emits the fluorescence of a fluorescence labeler molecule for the irradiation time of the light of each wavelength at this time although light is irradiated, i.e., S1, It is made shorter than the life of a condition. When it states qualitatively, it is wavelength lambda 1 first. It is light S1 of a fluorescence labeler molecule It irradiates between the time amount t sufficiently shorter than the life of a condition, and is S1 to an observation field. The molecule of a condition is made to generate. It is S1 as well as [observation] an unnecessary field immediately after that. Time amount wavelength lambda 2 sufficiently shorter than the life of a condition Light is irradiated and it is S1. It is a molecule in a condition S2 It excites in the condition and fluorescence is controlled. Hereafter, this process is explained quantitatively.
[0027] Generally, it is S0. It is wavelength lambda 1 about the molecule of a condition. It is S1 at light. When exciting in the condition, the rate equation shown below can describe the excitation process. That is, it

is the molecularity per [which dyed the sample] unit volume of a molecule N0 It carries out and is wavelength lambda 1. They are I0 and wavelength lambda 1 about the photon flux of light. It is S0 after time amount t from the exposure of light. Molecularity of a condition is set to N. And S1 The life of a condition is set to tau and it is wavelength lambda 1. It is S0 by light. A condition to S1 If the absorption cross section when changing in the condition is set to sigma01, this rate equation can be concretely expressed like the following equation (1).

$$-\frac{dN}{dt}=NI_0\sigma_{01}-\frac{(N_0-N)}{\tau} \quad \cdots\cdots(1)$$

[0028] Furthermore, if this formula (1) is solved, it will be wavelength lambda 1. It is S1 per unit volume after time amount t from the exposure of light. It can ask for the molecularity n of a condition.

$$n=\frac{N_0I_0\sigma_{01}\tau}{(1+I_0\sigma_{01}\tau)}\cdot\left[1-e^{\left\{-(I_0\sigma_{01}+\frac{1}{\tau})t\right\}}\right] \quad \cdots\cdots(2)$$

但し、 $n=N_0-N$

[0029] Moreover, it is wavelength lambda 1 so that it may be satisfied with the bottom of the conditions with which a formula (2) is filled of the conditions of the following formula (3). If light is irradiated, said formula (2) can be written like a formula (4) in approximation.

$$(I_0\sigma_{01}+\frac{1}{\tau})t\cong 0 \quad \cdots\cdots(3)$$

$$n\cong I_0\sigma_{01}N_0t \quad \cdots\cdots(4)$$

[0030] That is, according to the formula (3), it is wavelength lambda 1. It is the irradiation time of light S1 of a molecule It is made shorter than the life of a condition and, moreover, is wavelength lambda 1. When the photon flux of light is small, the molecularity n of S1 condition is proportional to irradiation time t mostly.

[0031] Next, wavelength lambda 1 The exposure of light is completed and it is wavelength lambda 2 immediately after that. S1 when irradiating light between time amount T It is a molecule in a condition S2 The case where it excites in the condition is considered. They are I1 and wavelength lambda 1 about the photon flux of the light of wavelength lambda 2. It is S1 after time amount (T+t) from the exposure of light. Molecularity of a condition is set to n and it is wavelength lambda 2. It is S1 by light. A condition to S2 When the absorption cross section when changing in the condition is set to sigma12, the rate equation about n is as follows.

$$\frac{dn}{dt}=-\sigma_{12}I_1n-\frac{n}{\tau} \quad \cdots\cdots(5)$$

[0032] By solving this formula (5), it is wavelength lambda 1. After only time amount t irradiates light, it is [a stop and] wavelength lambda 2 immediately after that. The value of said n at the time of irradiating light between time amount T can be expressed like a degree type (6).

$$n=(I_0\sigma_{01}N_0t)\cdot e^{-(\sigma_{12}I_1+\frac{1}{\tau})T} \quad \cdots\cdots(6)$$

On the other hand, it sets at a ceremony (6) and is wavelength lambda 2. When not irradiating light at all, it is I1 =0, $n=(I_0\sigma_{01}N_0t)\cdot e^{\frac{T}{\tau}}$ $\cdots\cdots(7)$

It becomes.

[0033] In fact, a formula (6) is S1 per [in the field which had fluorescence controlled] unit volume. It is S1 per [in the field to which the molecularity of a condition is shown and a formula (7) does not have fluorescence controlled] unit volume. The molecularity of a condition is shown. Fluorescence intensity F1 from a field which had fluorescence controlled when the fluorescence absorption coefficient of a molecule was set to phi Fluorescence intensity F2 from a field which does not have fluorescence controlled It is given by the following formula, respectively.

$$F_1=\Phi(I_0\sigma_{01}N_0t)\cdot e^{-(\sigma_{12}I_1+\frac{1}{\tau})T} \quad \cdots\cdots(8)$$

$$F_2=\Phi(I_0\sigma_{01}N_0t)\cdot e^{\frac{T}{\tau}} \quad \cdots\cdots(9)$$

[0034] Moreover, the fluorescence control ratio F1 / F2 It is expressed by a formula (8) and (9) like a degree type.

$$\frac{F_1}{F_2} = e^{-\sigma_{12} I_1 T} \quad \cdots\cdots (10)$$

[0035] Therefore, it is lambda 1 at the timing shown in drawing 1 . lambda 2 If the light of two kinds of wavelength is irradiated, the fluorescence from the field which is not needed for observation by the ratio shown by the formula (10) can be controlled. According to the formula (10), it is I1 at the conditions of T<tau. By adjusting T, fluorescence can be controlled by the ratio of arbitration.
[0036] The timing which observes the fluorescence intensity from an observation field is shown in drawing 2 . Fundamentally, the timing which measures fluorescence intensity is wavelength lambda 2. After the exposure of light is completed, the reinforcement of the fluorescence which emits light from an observation field will fully be measured over many hours. If it does in this way, it is in the condition that the fluorescence from the controlled field hardly exists, namely, the fluorescence from an observation field can be measured in the state of a very good S/N ratio.
[0037] Drawing 3 and 4 are lambda 1. lambda 2 It is drawing showing the timing which irradiates the light of two kinds of wavelength at a sample, and the timing which measures the reinforcement of the fluorescence which emits light from an observation field. Even if it goes by timing shown here, measurement of fluorescence can be performed effectively. However, the need has been. [with any / drawing 2 thru/or / which was shown four / case] [of the conditions of t and T<tau] Because, it is lambda 1 that they are t and T>tau. lambda 2 It is S1 while irradiating the light of two kinds of wavelength. The molecule of a condition is S0. It is because deexcitation is changed into a condition and the fluorescence from an observation field itself is lost. It is lambda 1 as it is temporarily shown in drawing 5 , since it corresponds in t and T>tau. lambda 2 Although the approach of measuring the reinforcement of the fluorescence which irradiates the light of two kinds of wavelength at coincidence, and emits light from an observation field to coincidence is also possible, it is lambda 1 in this case at the time of fluorometry. lambda 2 There is a possibility that a strong excitation light of two kinds of wavelength may mix in a detector. Therefore, it is lambda 1 at the timing shown in drawing 2 thru/or 4 under the conditions of t and T<tau. lambda 2 It is desirable to irradiate the light of two kinds of wavelength at a sample.
[0038] Moreover, at this invention, it is lambda 1. lambda 2 Although the fluorescence which emits light from an observation field must be measured with a detector from from immediately after exposure ending to the sample of the light of two kinds of wavelength, in that case, a commercial general-purpose logical circuit generates a gate signal, and the activity which incorporates the electrical signal outputted from a detector in the memory of a personal computer is needed. In case the approach of this invention is performed like the timing diagram shown in drawing 1 thru/or 4 at this time, it is effective if it is made for the irradiation time of the light to a sample to become shorter than the life time of fluorescence of the molecule which a sample is dyed. However, in the general-purpose logical circuit by which current marketing is carried out, since the switching rate is about 1ns, tau itself is wanted to be 1ns or more. If tau is not 1ns or more, before a detector and a measurement circuit will become active, it is because the fluorescence phenomenon from an observation field is completed (Japanese Texas Instruments, Inc.: Texas Instruments ALS/AS advance DOBAIPORAROJIKKU family data book (1991)). In consideration of the above situation, the conditions that it must have the life time of fluorescence for 1ns or more will be added to the fluorescence labeler molecule which a sample is dyed.
[0039] Furthermore, although the one where fluorescence intensity is weaker is desirable in a fluorescence control field to be sure when its attention is paid to the effective fluorescence field for extracting measurement data, from a viewpoint of improvement in a S/N ratio, the strong one of the luminescence reinforcement of an effective fluorescence field is desirable. Namely, wavelength lambda 1 S1 [immediately after exciting with light] It is desirable to measure the fluorescence intensity in the time of day when the molecularity of a condition exists enough. According to said formula (9), the number of excited molecules is exponentially decreased with the time constant which becomes settled from the excitation life.
[0040] By the way, the irradiation time t and T of light is S1 as a property of an exponential function. If shorter enough than the life time of fluorescence tau of the molecule of a condition, it is wavelength lambda 1. S1 [immediately after exciting with light] The fluorescence of sufficiently strong reinforcement, i.e., valid signal reinforcement, can be measured from the molecule of a condition. Especially, t and T are S1. It is S1 if it is about [of the life time of fluorescence tau of the molecule of a condition] 1/10. Since there is molecularity of a condition no less than 90% of the molecularity immediately after exciting with the light of wavelength lambda 1, sufficient signal strength is obtained from an effective fluorescence field.

[0041] Hereafter, this invention is explained to a detail based on the illustrated example.
[0042] A 1st example epsilon-adenosine (1, N6-ethenoadenosine) is a typical fluorescence labeler, and introduces the observation approach by the scanning fluorescence microscope which used this molecule as a fluorescent probe molecule in this example.
[0043] It is proved by many enzyme systems that epsilon-adenosine works as a good labeler molecule in which the amount of distribution of ATP or ADP is shown in the form (epsilon-ATP and epsilon-ADP) of NUKURECHIO. On the other hand, if it thinks as a fluorescent probe molecule, it will become an advantage that a fluorescence yield is high, that excitation wavelength does not lap with a nucleic acid or protein, etc., when applying to a biological material. Furthermore, fluorescence wavelength and a fluorescence yield have the property which moves to a short wavelength side as the maximum wave length of fluorescence becomes large, although the polarity of a solvent etc. does not receive change (Japan Scientific Societies Press "fluorometry (1933) Kazuhiko Oshita / ******"). For this reason, when applying to protein etc. by using epsilon-adenosine as a fluorescent probe molecule, it is shown that firefly luminescence wavelength can serve as whenever [restraint / of a NUKURECHIODO binding site], and an environmental viscous index.
[0044] epsilon-adenosine is available in many fields, such as discernment of the structural change of an enzyme based on change of a fluorescence spectrum taking advantage of the above point, the structural change near the active center of an actin or a myosin based on observation of energy transfer, and the NUKURECHIODO binding site of the green leaf object coupling factor by degree of polarization or fluorescence disappearance. Moreover, it has the physical property as shown in the next table 1, the excitation life of S1 condition is very long especially, and epsilon-adenosine is 20sec in the water solution of pH7.0. It has the life time of fluorescence. Therefore, epsilon-adenosine is used as a fluorescent probe molecule, this dyes a biological material, and if it observes using the super resolution microscope using a double resonance absorption process, detailed chemical composition analysis of a NUKURECHIODO binding site will be attained with resolving power between altitude. Hereafter, this example describes the case where epsilon-adenosine is used as a fluorescent probe molecule.
[0045]
表1 ε－アデノシンの物理特性

分子量	327．73
溶解性／溶媒	水溶性
蛍光寿命	20nsec
蛍光収率	0．56
最大吸収波長	294nm
最大蛍光波長	415nm

[0046] First, the principle of the super resolution microscope which used the double resonance absorption process in which it was used for this approach, as a premise explaining the observation approach of concrete this example is explained. Drawing 6 R> 6 (a) is drawing showing the configuration of the optical system which constitutes the microbeam which is the main component of the super resolution microscope using a double resonance absorption process. Here, it is lambda 1 from the same point. lambda 2 The light source 1 to which the spectrum of the light which has wavelength is carried out, and it emits light is assumed. The wavelength lambda 1 emitted from this light source 1, and lambda 2 Light irradiates the aperture 2 which has zona-orbicularis structure. The light which escaped from the aperture 2 is lambda 1. lambda 2 By the optical system 3 equipped with a condensing location which is different in a wavelength band, it is wavelength lambda 1. It is condensed on the sample side 4 for the purpose of observation, and light is wavelength lambda 2. Light irradiates the sample side 4 top in the condition of having been defocused. At this time, the sample is carried on the stage in which the 2-dimensional scan within the image formation side of optical system 3 and the scan of the direction of an optical axis are possible (un-illustrating). Moreover, you may make it scan the beam itself, using a scanning mirror etc. as an approach of scanning a 2-dimensional sample by the microbeam.
[0047] Moreover, the aperture 2 which has the zona-orbicularis structure where it is used here is equipped with the following special configurations. That is, although drawing 6 (b) is the front view showing the configuration of an aperture 2, the aperture 2 has duplex zona-orbicularis structure of a field R1 (center

section) and a field R2 (zona-orbicularis section). Field R1 among duplex zona-orbicularis structures Wavelength lambda 1 Although it has sufficient permeability to light, it is wavelength lambda 2. To light, it has only very low permeability. On the other hand, it is a field R2. Wavelength lambda 2 Although it has sufficient permeability to light, it is wavelength lambda 1. There is a property to have only very low permeability to light.

[0048] The aperture 2 equipped with such a description can be easily manufactured, if a vacuum evaporationo technique is used. For example, a zona-orbicularis mask is formed on the quartz plate which has high permeability in the large wavelength range including visible and an ultraviolet region, and it is a field R1. Wavelength lambda 1 It has sufficient permeability to light and is wavelength lambda 2. The quality of the material which has only very low permeability to light, and dielectric multilayers are vapor-deposited. on the other hand, field R2 **** -- wavelength lambda 2 light -- receiving -- sufficient permeability -- having -- wavelength lambda 1 The quality of the material which has only very low permeability to light, and dielectric multilayers are vapor-deposited. In addition, it is necessary to choose appropriately selection of the quality of the material to vapor-deposit or dielectric multilayers according to the wavelength band of the light to be used.

[0049] Moreover, at drawing 6 (a), it is wavelength lambda 1. Wavelength lambda 2 The image formation pattern on the sample side 4 of light (intensity distribution) is also illustrated notionally. the inside of drawing, and a field 5 -- wavelength lambda 1 The image formation pattern of pump light is shown. Moreover, a field 6 is wavelength lambda 2. It is an image formation pattern and has become what is depended on having made it defocus by the aperture 2, i.e., the so-called inside omission pattern. Therefore, according to the principle of this invention, it is wavelength lambda 1 as mentioned above. Since, as for the part with which the light of wavelength lambda 2 lapped, fluorescence is controlled, a fluorescence field is limited to the part of a field 7 (slash section), and it is wavelength lambda 1. Only a field smaller than the field where light is irradiated will emit fluorescence. Thereby, the spatial resolving power of a microscope can be raised exceeding the diffraction marginal condition by wavelength.

[0050] Although it is drawing showing the configuration of the optical system which, on the other hand, constitutes the microbeam whose drawing 7 is also the main component of the super resolution microscope using a double resonance absorption process, they are wavelength lambda 1 and wavelength lambda 2 here. The case where light is emitted from the light sources 1a and 1b in the location from which light differs on the same optical axis is shown. Also in this case, it is wavelength lambda 1. And wavelength lambda 2 The light which light irradiated the aperture 2 which has zona-orbicularis structure, and escaped from the aperture 2 is lambda 1. lambda 2 It is condensed on the sample side 4 by the optical system 3 equipped with the same condensing location (image formation property) in the wavelength region. At this time, the sample is carried like what was shown in drawing 6 (a) on the stage in which the 2-dimensional scan within the image formation side of optical system 3 and the scan of the direction of an optical axis are possible (un-illustrating). Moreover, it is the same as that of that the configuration of an aperture 2 was also indicated to be to drawing 6 (b). However, in the case of the microscope shown in drawing 7 , since light equipment itself is in the location where it differs on an optical axis, it is wavelength lambda 2. Light irradiates the sample side 4 in the condition of having been defocused, and the same image formation pattern (intensity distribution) as what was shown in drawing 6 (a) as a result is formed on the sample side 4. At this time, optical system 3 is lambda 1. lambda 2 Although it is required to have the same condensing location (image formation property) in the wavelength field, in current lens engineering, it can design, if optical system with the lens configuration of two or more sheets is used, and the catoptric system which chromatic aberration does not generate theoretically may be used.

[0051] Next, the concrete system configuration Fig. of the scanning fluorescence microscope used in order to perform the observation approach of this example is shown in drawing 8 . Here, the system which combined two sets of Nd:YAG laser 11 and dye laser 12 and 13 as the light source is considered as a fluorescent probe molecule supposing epsilon-adenosine. The 1st coloring matter laser system is dye laser 12 and BBO. It consists of a 2 double wave oscillator 14 which is a crystal. Nd: -- 3 time wave (355nm) of YAG laser 11 the dye laser 12 which considers as excitation light and uses a coumarin as a medium -- 400-600nm the light of a wavelength field is oscillated -- making -- further -- 2 double wave oscillator 14 -- this light -- 200-300nm It changes into the light of a wavelength field. this light -- epsilon-adenosine -- ground state S0 from -- S1 It is excited by the condition.

[0052] Moreover, Nd: It branches with a half mirror 15 and let the 3 time wave of YAG laser 11 be the excitation light of the dye laser 13 as 2nd coloring matter laser system. It is 400-600nm by choosing a coumarin also as a solvent of dye laser 13 like dye laser 12. The light of a wavelength field is oscillated. To

this wavelength field, it is S1. They are 6 membered-ring molecules of a condition S2 It is S1 also about epsilon-adenosine which contains 6 membered-ring molecules since the resonance wavelength excited in the condition exists (97 H. Kandori, et.al.J.Phys.Chem. 9664 (1993)). A condition to S2 It can excite in the condition. In addition, two mirrors 16 and 17 are formed in the optical path according to which dye laser 13 is arranged.

[0053] Thus, the light of two oscillated colors is doubled on the same optical axis with a dichroic mirror 18. The light of two compounded colors is made to condense with the 1st condenser lens 19 of a two-wave band, and the aperture 20 which has zona-orbicularis structure is irradiated. The configuration of this aperture 20 is the same as that of the aperture 2 shown in drawing 6 (b). Moreover, when color correction of the condenser lens 19 of a two-wave band is carried out in this two-wave band at this time, in order to reduce the laser noise by the unnecessary diffracted light and unnecessary dispersion, it is desirable to arrange the spatial filter 21 as a pinhole in the condensing location of the 1st condenser lens 19.

[0054] The flux of light by which the spectrum was carried out by the aperture 20 is condensed on the biological material 24 currently laid on the stage 23 which can be scanned in the direction of an arrow head of drawing with the 2nd condenser lens 22 of the two-wave band which consists of image formation lens 22a and objective lens 22b. The fluorescence (wavelength lambda 3) which epsilon-adenosine in a biological material 24 emits is the wavelength lambda 1 arranged between image formation lens 22a and objective lens 22b, and lambda 2. Light is penetrated and it is wavelength lambda 3. It is reflected with the dichroic mirror 25 which reflects light, and is detected by the photograph mull 27 through the band pass filter 26 which penetrates only fluorescence. At this time, the 3rd condenser lens 28 is arranged between a dichroic mirror 25 and the PANDO pass filter 26, a pinhole 29 is arranged in the condensing location of this 3rd condenser lens 28 again, and it gets down, and is wavelength lambda 3. Only that to which fluorescence penetrated the pinhole 29 will be detected by the photograph mull 27.

[0055] In order to attain the so-called confocal observation of it since the scanning fluorescence microscope used in this example is constituted in this way, and to have resolution also in the depth direction (the direction of an optical axis), three-dimensions observation is attained. In addition, since a more effective signal is detectable by setting up the magnitude of the path of said pinhole 29 appropriately, it is S/N. Improvement in a ratio can be aimed at. Moreover, in order to scan a stage 22 by the below-mentioned approach down the stage 23, the photograph mull 30 is formed.

[0056] By the way, in order to form the image formation pattern (intensity distribution) of the light of each wavelength in a sample side so that it may become being the same as that of the thing of the microscope shown in drawing 6 (a), you may constitute so that it may have a condensing location which is different for every wavelength in the 2nd condenser lens 22 of a two-wave band. Moreover, when [like / (chromatic aberration is amended)] the 2nd condenser lens 22 condenses the light of two different wavelength in the same location, the 1st condenser lens 19 may be equipped with the function of chromatic aberration, the condensing location of the laser light of each wavelength may be shifted, and you may change into the condition that it is shown in drawing 7 . However, in the case of the latter, since the condensing locations of each laser light by the 1st condenser lens 19 differ, a spatial filter 20 cannot be arranged in a condensing location.

[0057] Moreover, Nd which has a comparatively long pulse for about 7ns as the excitation light source in this example: Although YAG laser 11 is used, since epsilon-adenosine as a fluorescent probe molecule has the still longer life time of fluorescence for about 20ns, it is satisfactory. Furthermore, in this example, for example, the titanium sapphire laser other than the light source which combined Nd:YAG laser 11 and dye laser 12 and 13 may be used, and the semiconductor laser of CW (continuous oscillation) can also be used. Moreover, wavelength adjustable optical parametric oscillator used well recently (OPO) Laser system is also employable. It is not necessary to say that it is naturally possible to make laser system based on the titanium sapphire laser of the mode locking of the short pulse for 1 or less ns or Nd:YAG laser into the light source.

[0058] Moreover, in order to pile up two waves of light of the light source spatially about an aperture 21, the thing equipped with zona-orbicularis structure was adopted, but since what is necessary is just the structure which an optical pupil separates wavelength-wise and spatially and can penetrate fundamentally, the coat of the quality of the material or dielectric multilayers vapor-deposited by the aperture 2 shown on the front face of the 2nd condenser lens 22 at drawing 6 (b), for example may be carried out.

[0059] Here, in the observation approach of this example, the fluorescence control ratio (F1 / F2) at the time of choosing epsilon-adenosine as a fluorescent probe molecule is calculated by the above-mentioned formula (10). They are six membered-rings S1 A condition to S2 Absorption cross section sigma12 when exciting in the condition are 10-17 cm2. It is extent (97 H. Kandori, et.al.J.Phys.Chem. 9664 (1993)). And

they are 7ns and photon flux about the pulse width of the dye laser 12 and 13 which is the light source (400-600nm field in wavelength lambda2 : this case) for controlling fluorescence 5x1025 photons/sec/cm2 If it carries out, a fluorescence control ratio will be set to 0.03. That is, the fluorescence intensity of the part by which the light of wavelength lambda 2 was irradiated will be controlled by 3/100 of the part by which it is not irradiated. Moreover, although it is equivalent to 25 MW/cm2 when the value of said photon flux is changed into laser reinforcement, the fluorescence microscope of a laser scanning-type using current and an off-resonance two-photon absorption process is put in practical use. However, in order that laser reinforcement may also attain to number TW/cm2 in the case of this microscope (Kkong, et.al.Opt.Lett.15,135 (1997)), as compared with this, the reinforcement of the laser in the case of this example is understood that it is low 4 or more figures, and the damage to a sample is also far small.

[0060] Drawing 9 is a system chart of an electric system which ****s to the microscope system shown in drawing 8 . In here, the photograph mull 30 acts as the monitor of the light from each laser light source, and the photograph mull 27 acts as the monitor of the fluorescence from a sample 24. Although the output signal from the photograph mull 27 becomes data which are 1 pixel of the fluorescence image of a sample 24 fundamentally, it acts as the monitor of the quantity of light of the light which penetrated the sample 24 in every shot of the laser light as illumination light.

[0061] Moreover, the whole system of this microscope system is controlled by the personal computer 31. This personal computer 31 incorporates the signal from two photograph mull 27 and 30, and performs drive control of the stage 23 in which an oscillation and sample 24 of Nd:YAG laser 11 are laid. All the timing of a system is based on the clock of a personal computer 31, and carries out dividing of this clock with a counting-down circuit 32 to the frequency in which laser oscillation is possible. Delay and wave-like amendment are carried out for the clock signal by which dividing was carried out here with gate - and - delay generator 33, it considers as the Q switch signal and flash lamp signal for laser control, and Nd:YAG laser 11 is controlled. A current / electrical-potential-difference conversion is carried out by pre amplifier 34 and 35, and the output signal from two photograph mull 27 and 30 is further stored in the frame memory of a personal computer 31 as numeric data by A/D converters 36 and 37.

[0062] Moreover, the full synchronization is carried out with luminescence of laser as the timing of incorporation controlled by the output signal of gate - and - delay generator 33 and shown in drawing 1 thru/or 5 to A/D converters 36 and 37 of the output signal from the photograph mull 27 and 30, and the personal computer 31 of data. Moreover, a personal computer 31 also performs control of a stage 23, it synchronizes luminescence of laser, and incorporation of data, and can be scanned now two dimensions. Thus, the generated image data is outputted by CRT38 or the video printer 39.

[0063] As mentioned above, although the example using epsilon-adenosine as a fluorescent probe molecule was shown, in this example, other matter may be used as a fluorescent probe molecule. For example, the molecule shown below all has the very long life time of fluorescence and a large fluorescence yield including six membered-rings or a double bond, and can apply them to the super resolution microscope using a double resonance absorption process.

[0064] 4-Fluoro-7-sulfamoylbenzofurazan:3,6-Bis(dimethylamino)-10-dodecylacridinium bromide:4-Benzylamino-7-nitrobenzofurazan: 4-Azidofluorescein diacetate,5,6-Dimethoxy-2- (4-hydrazinocarbonylphenyl)benzothiazole:3-Bromomethyl,6,7-dimethoxy-1-methyl-1,2-dihydroquinoxaline-2-one : 4-Bromoethyl-7-methoxycoumarin:N-[-- 4- (it dimethoxybenzene(s) 6-Dimethylamino-2-benzofurany]maleimide:1, 2-Diamino-4, and 5-) dihydrochloride: 2, 2'-Dihydroxy-6, and 6 '-dinaphthyl disulfide:3-Chlorocarbonyl-6, 7-dimethoxy-1-methyl-2(1H)-quinoxalinone:2-2'-Dithiobis (1-aminoaphthalene), Fluorescein-4-isothiocyanate:4-Amino-3-penten-2-one:4-(4-Methoxybenzylamino)-7-nitrobenzofurazan:N-[4-]maleimide:1, 2-Diamino-4, and 5-methylenedioxybenzene:N-(9-Acridinyl) maleimide : (5, 6-Methylenedioxy-2-benzofuranyl) 4-Fluoro-7-nitrobenzofurazan, 4-Chloro-7-nitrobenzofurazan:(S)-(-)-1 - (2) [3-Napthalenedicarboximidyl fluoride:2-] (5-Chlorocarbonyl-2-oxazolyl) - 5, 6-methylenedioxybenzofuran:4-Chloro-7-sulfobenzofurazan, ammonium salt: 4-Fluoro-7-sulfobenzofurazan, ammonium salt:Sulforthodamine 101 acid chloride:4-[4-(Dimethylamino) phenylazo] phenylisothiocyanate:3 and 5-Dinitrobenzoyl chloride: 5- (4-Dimethylaminophenyl) -2, 4-pentadienal:1, 3-Diphenyl-2-thiobarbituric acid:O-(4-Nitrobenzyl)-N, and N'-diisopropylisourea:O- hydroxylamine, hydrochloride:N, N, and N'-Triethyl-N'-[N-(N-succinimidyloxycarbonyl) pentyl]-9-cyanopyronine (4-Nitrobenzyl) chloride:N, N, and N'-Triethyl-N'- {5-[N"- piperazinocarbonyl]pentyl}9-cyanopyronine chloride:2, (2-maleimidoethyl) 4'-Dibromoacetophenone:N-Succinimidyl-4-nitrophenylacetate : [0065] O,O'-Bis (2-aminophenyl)ethyleneglycol-N,N,N',N'-tetraacetic-acid,tetraapotassium,hydrate:O,O'-Bis(2-aminophenyl)ethyleneglycol-N, N,N',N'-tetraacetic acid,tetraacetoxymethyl ester:O,O'-Bis (2-amino-5-

fluorophenoxy)ethyleneglycol-N, N,N',N'-tetraacetic acid, tetraapotassium, hydrate: O, O' - Bis ethyleneglycol-N, N, N', N'-tetraacetic acid, tetraacetoxymethyl ester:N, N, N', N'-Tetrakis (2-amino-5-fluorophenoxy) (2-pyridylemethyl) ethylenediamine:1-[2-amino-5- (2 --) 7-dichloro-6-hydroxy-3-oxy-9-xantheny]-2-(2-Amino-5-methylphenoxy) ethane-N, N, N', N'-tetraacetic acid:1-[2-Amino-5-]-2-(2-amino-5-methylphenoxy) ethane-N, N, N', N'-tetraacetic acid, (2 7-dichloro-6-hydroxy-3-oxy-9-xantheny) pentaacetoxymethyl es tar:1-[6-Amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid,pentapotassium salt:1-[6-Amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid and pentaacetoxymethyl ester:1-[6-Amino-5-(6-carboxy-2-indolyl) phenoxy]-2-(2-amino-5-methylphenoxy) ethane-N, N, N', and N' -tetraacetic acid and pentapotassium salt:1-[6-Amino-5-(6-carboxy-2-indolyl) phenoxy]-2-(2-amino-5-methylphenoxy) ethane-N, N, N', and N' -tetraacetic acid and pentaacetoxymethyl ester -- : -- eight - Amino - two - [-- two - amino - five - methylphenoxy -- methyl --] - six - methoxyquinoline-N -- N -- N -- ' -- N -- ' - tetraacetic -- tetrapotassium salt -- : -- eight - Amino - two - [(2-amino-5-methylphenoxy) -- methy -- one --] - six - methoxyquinoline-N -- N -- N -- ' -- N -- ' - tetraacetic -- tetraacetoxymethyl ester: 1-[(2-Amino-5- ()) [3-dimethylamino-6-dimethylammonio-9-xanthenyl]-2-(2-amino-5-methylphenoxy) ethane-N, N, N',] [N'-tetraacetic] acid, chloride:1-[(2-Amino-5- ()) [3-dimethylamino-6-dimethylammonio-9-xanthenyl]-2-(2-amino-5-methylphenoxy) ethane-N, N, N',] [N'-tetraacetic] acid, tetraacetoxymethyl ester, chloride:2'.7'-Bis(carboxyethyl)-4 or 5-carboxyfluoreescein: [0066] 3'-O-Acetyl-2',7'-bis (carboxyethyl)-4 or 5-carboxyfluorescien,diacetoxymethyl-ester:8-Amino-2-(trans-2-aminostyryl)-6-methoxyquinoline-N,N,N',N'-tetraacetic acid,tetrapotassium salt:8-Amino-2-(trans-2-aminostyryl)-6-methoxyquinoline-N,N,N',N'-tetraacetic acid and tetraacetoxymethyl ester:4 and 4'-Bis[-- {-- 6-[N, N-bis(2-hydroxyethyl) amino]-4-phenylamino- 1, 3, 5-triazin-2-yl}amino]-2, and 2' -stilbenedisulfonic acid, disodium salt:4.4' - Bis[{-- 6-[N, N-diethylamino]-4-phenylamino- 1, 3, 5-triazin-2-yl}amino]-2, and 2'-stilbenedisulfonic acid -- disodium salt:4, 4'-Bis[(6-methoxy-4-phenylamino- 1, 3, 5-triazin-2- yl) amino]-2, 2'-stilbenedisulfonic acid, and disodium salt: 1, 3-Bis propane, 1- (1-pyrenyl) (4-Trimethylammoniumphenyl)-6-phenyl-1, 3, 5-hexatrieneiodide:N-[3-(1, 5-Disulfonaphtyl)]-N'-[4-(2, 2, 6, and 6-tetramethylpiperidine-N-oxide) thiouurea, and disodium salt:N-Ethoxycarbonylmethyl-6-methoxyquinolinium promide:6-Methoxy-N-(3-sulfopropyl) quinolinium, monohydrate:5-or-6- (N-Succinimidyloxycarbonyl)-3' and 6 -- '-diacetylfluorescein:5-or-6-(N-Succiniumidyloxycarbonyl)-4' and 5 '-diamethyl-3', 6'-O -- O'-diacetylfluorescein:5-or-6-(N-Succiniumidyloxycarbonyl)-4', 5'-dichloro-3', 6' - O, O' - diacetylfluorescein: [0067] 3,6-bis-dimethylaminoacridine:9-aminoacridine:9-(4-diethylamino-methylbutylamino)-3-chloro-7-methoxyacridine:1-anilinonaphthalene-8-sulfonate:N-methyl-2-anilinonaphthalene-6-sulfonate:2-p- toluidinyl-naphthalene-6-sulfonate:12(9-anthroyloxy)stearic-acid:teramethyldiaminodiphenylketoimine-hydrochloride: 7-chloro-4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy:cyanine-dyes:1,1'-dihexyl-2, 2'-oxacarbocyanine: 3, 3'-dipropylthiadicarbocyanine: 5-[() [3-sulfopropyl-2] (3H)-benzoxazolylidene-2-butenylidene] -1, 3-dibutyl-2-thiobarbituricacid:5-dimethylaminonaphthalene-1-sulfonamide : dansylamino-ethyltriphosphate: 1-(5-dimethylaminonaphthalene-1-sulfonamide)-3-N, N, -dimethyaminopropane:1- (5-dimethylaminonaphthalene-1-sulfonamido)-propane-3-trimethlammonium:(N-dansyl)-aminoalkyl- Beta-D-galactopyranoside: Epsilon-dansyl-L-lysine : N6-ethennoadenosine: dansylphosphaidyl-ethanolamine:1 and 6-diphenyl- 1, 3, and 5-hexatriene:2' -- 4', 5', and 7'-tetrabromofluorescein:1 -- 2 7-diamino-9-phenylphenanthrium-10-ethyl-bromide:9-(o-carboxyphenyl)-6-hydroxy-3 H-xanthen-3-one:[0068] 7-amino-3- pyrazolo (Beta-D-ribrofuranosyl) pyrimidine: (4, 3-d) 4-benzoylamido-4 -- '-aminostilbene-2-2' -disulfonate:1-acyl-2-[N- (4-nitrobenzo-2-oxa-1 --) 3-diazolylaminocaproyl]phosphatridylcholin e:beta-naphthyltriphosphat e:oxonol dye:bis[3-phenyl-5-oxoisoxazol-4-yl] pentamethineo xonol:bis[1 and 3-dibutylbarbituric-acid (5)] pentamethineoxonol: alpha(9, 11, 13, 15-cis.trans, trans, cis) octadecatetraenoic Acid:beta It all(s). 9, 11, 13, and 15- transoctadecateraenoic acid:perylene:N-phenyl-1-napthylamine:pyrene:2, 3-dimethyl-3, 7-diamino-5-phenylphenazium:4-phenylspro[furan-2 (3H), 1'-futalan] -3 and 3'-dione:o-phthalic dicarboxaldehyde:1-dimethylaminonaphthalene-5-sulfonyl chloride:flurorescien isothiocyanate: 7-chloro-4-nitrobenzo-2-oxal, 3-diazole:N-dansyl aziridine:5-(iodoacetoamidoethyl) amino-naphthalene-1-sulfonate : 5-iodoacetamido fluorescein:N- maleimide:N-(7-dimethylamino-4-methylcoumarynul) maleimide:N-(3-pyrene) maleimide:eosin-5-i (1-anilinonaphthyl-4) odoacetamide:fluorescein mercuty-acetate: 2-[4'(2"-iodoacetamido) pheyl] aminonaphthalene-6-sulfonic acid: [0069] among these, o-phthalaldehyde (o-phthalic dicarboxaldehyde) etc. -- since the strong fluorescence intensity stabilized when it was in the solution of pH 6-11.5 is maintained, many fluorescent probes can form a pixel with the stable signal strength, and can expect a good microscope image. Therefore,

if the dyed sample is dipped and measured in the buffer solution of pH 6-11.5, the high-definition image which harnessed the advantage of this invention enough will be obtained.
[0070] 2nd example drawing 10 is drawing showing the configuration of the scanning fluorescence microscope used by the observation approach of this example. In addition to the configuration of the microscope which showed the scanning fluorescence microscope shown here to the 1st example, two polarizers 41 and 42 are arranged at the injection side of 2 double wave oscillator 14 and dye laser 13, respectively. Thus, the plane of polarization of the laser light oscillated from two sets of dye laser can be controlled now independently by having polarizers 41 and 42, respectively. And since there is a correlation with strong plane of polarization of light and direction of luminous intensity distribution of the molecule which a sample 24 is dyed, when each plane of polarization of the light of two wavelength and the direction of orientation of said molecule make a specific include angle, the information on spatial orientation distribution of a sample 24 is acquired from change of extent of disappearance of fluorescence by using that a double resonance absorption process arises.
[0071] In case the light of two wavelength is irradiated on a sample 24 at coincidence, specifically, the plane of polarization of each laser light is set up by making two polarizers 41 and 41 penetrate. Since the plane of polarization of two laser light supports the spatial orientation of the molecule which a sample 24 is dyed at this time, only when specific polarization conditions are fulfilled, double resonance absorption is produced strongly and the fluorescence from said molecule which carried out orientation in the specific direction corresponding to this disappears. From the decrement of this fluorescence, analysis of the condition of the space orientation of the sample 24 to observe can be performed. Thus, in addition to super resolution nature, space orientation analysis of the organization of a sample can also be performed by the observation approach by this example.
[0072] As explained above, the observation approach of the microscope of this invention was combined with the description indicated by the claim, and is equipped also with the description as shown in (1) - (10) below.
[0073] (1) The observation approach of the microscope according to claim 1 characterized by using the molecule which has a double bond for dyeing of a sample.
[0074] (2) The observation approach of the microscope according to claim 1 characterized by using the molecule which includes one at least 6 membered-rings as a chemical group for dyeing of a sample.
[0075] (3) The observation approach of the microscope according to claim 1 characterized by using any of the molecule shown below they are, and dyeing a sample.
4-Fluoro-7-sulfamoylbenzofurazan:3,6-Bis(dimethylamino)-10-dodecylacridinium bromide:4-Benzylamino-7-nitrobenzofurazan: 4-Azidofluorescein diacetate,5,6-Dimethoxy-2- (4-hydrazinocarbonylphenyl) benzothiazole:3-Bromomethyl,6,7-dimethoxy-1-methyl-1,2-dihydroquinoxaline-2-one : 4-Bromoethyl-7-methoxycoumarin:N-[-- 4- (it dimethoxybenzene(s) 6-Dimethylamino-2-benzofurany]maleimide:1, 2-Diamino-4, and 5-) dihydrochloride: 2, 2'-Dihydroxy-6, and 6 '-dinaphthyl disulfide:3-Chlorocarbonyl-6, 7-dimethoxy-1-methyl-2(1H)-quinoxalinone:2-2'-Dithiobis (1-aminoaphthalene), Fluorescein-4-isothiocyanate:4-Amino-3-penten-2-one:4-(4-Methoxybenzylamino)-7-nitrobenzofurazan:N-[4-] maleimide:1, 2-Diamino-4, and 5-methylenedioxybenzene:N-(9-Acridinyl) maleimide : (5, 6-Methylenedioxy-2-benzofuranyl) 4-Fluoro-7-nitrobenzofurazan, 4-Chloro-7-nitrobenzofurazan:(S)-(-)-1 - (2) [3-Napthalenedicarboximidyl fluoride:2-] (5-Chlorocarbonyl-2-oxazolyl) -5, 6-methylenedioxybenzofuran:4-Chloro-7-sulfobenzofurazan, ammonium salt: 4-Fluoro-7-sulfobenzofurazan, ammonium salt:Sulforthodamine 101 acid chloride:4-[4-(Dimethylamino) phenylazo] phenylisothiocyanate:3 and 5-Dinitrobenzoyl chloride: 5- (4-Dimethylaminophenyl) -2, 4-pentadienal:1, 3-Diphenyl-2-thiobarbituric acid:O-(4-Nitrobenzyl)-N, and N'-diisopropylisourea:O- hydroxylamine, hydrochloride:N, N, and N'-Triethyl-N'-[N-(N-succinimidyloxycarbonyl) pentyl]-9-cyanopyronine (4-Nitrobenzyl) chloride:N, N, and N'-Triethyl-N'- {5-[N"- piperazinocarbonyl]pentyl}9-cyanopyronine chloride:2, (2-maleimidoethyl) 4'-Dibromoacetophenone:N-Succinimidyl-4-nitrophenylacetate:O and O'-Bis (2-aminophenyl) ethyleneglycol-N, N, N', N'-tetraacetic acid, tetraapotassium, hydrate:O, O'-Bis(2-aminophenyl) ethyleneglycol-N, N, N', N'-tetraacetic acid, and tetraacetoxymethyl ester:O, O' - Bis ethyleneglycol-N, (2-amino-5-fluorophenoxy) N, N', and N'-tetraacetic acid, tetraapotassium, hydrate: O, O' - Bis ethyleneglycol-N, N, N', N'-tetraacetic acid, tetraacetoxymethyl ester:N, N, N', N'-Tetrakis (2-amino-5-fluorophenoxy) (2-pyridylemethyl) ethylenediamine:1-[2-amino-5- (2 --) 7-dichloro-6-hydroxy-3-oxy-9-xantheny]-2-(2-Amino-5-methylphenoxy) ethane-N, N, N', N'-tetraacetic acid:1-[2-Amino-5-]-2-(2-amino-5-methylphenoxy) ethane-N, N, N', N'-tetraacetic acid, (2 7-dichloro-6-hydroxy-3-oxy-9-xantheny) pentaacetoxymethyl estar: 1-[6-Amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-

methylphenoxy) ethane-N, N, N', N'-tetraacetic acid,pentapotassium salt:1-[6-Amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid,pentaacetoxymethyl ester:1-[6-Amino-5-(6-carboxy-2-indolyl)phenoxy]-2-(2-amino-5-methylphenoxyethane-N, N, N', and N'-tetraacetic acid and pentapotassium salt:1-[6-Amino-5-(6-carboxy-2-indolyl) phenoxy]-2-(2-amino-5-methylphenoxy) ethane-N, N, N', and N' -tetraacetic acid and pentaacetoxymethyl ester -- : -- eight - Amino - two - [-- two - amino - five - methylphenoxy -- methyl --] - six - methoxyquinoline-N -- N -- N -- ' -- N -- ' - tetraacetic -- tetrapotassium salt -- : -- eight - Amino - two - [(2-amino-5-methylphenoxy) -- methy -- one --] - six - methoxyquinoline-N -- N -- N -- ' -- N -- ' - tetraacetic -- tetraacetoxymethyl ester: 1-[(2-Amino-5- ()) [3-dimethylamino-6-dimethylammonio-9-xanthenyl]-2-(2-amino-5-methylphenoxy) ethane-N, N, N',] [N'-tetraacetic] acid, chloride:1-[(2-Amino-5-()) [3-dimethylamino-6-dimethylammonio-9-xanthenyl]-2-(2-amino-5-methylphenoxy) ethane-N, N, N',] [N'-tetraacetic] acid and tetraacetoxymethyl ester -- chloride:2'.7'-Bis(carboxyethyl)-4 or 5-carboxyfluoreescein:3'-O-Acetyl-2', 7'-bis(carboxyethyl)-4 or 5-carboxyfluorescien, diacetoxymethyl ester:8-Amino-2-(trans-2-aminostyryl)-6-methoxyquinoline-N, N, N', and N'-tetraacetic acid and tetrapotassium salt:8-Amino-2-(trans-2-aminostyryl)-6-methoxyquinoline-N, N, N', N'-tetraacetic acid, and tetraacetoxymethyl ester:4 and 4'-Bis[-- {-- 6-[N, N-bis(2-hydroxyethyl) amino]-4-phenylamino- 1, 3, 5-triazin-2-yl}amino]-2, and 2' -stilbenedisulfonic acid, disodium salt:4.4' - Bis[{-- 6-[N, N-diethylamino]-4-phenylamino- 1, 3, 5-triazin-2-yl}amino]-2, and 2'-stilbenedisulfonic acid -- disodium salt:4, 4'-Bis[(6-methoxy-4-phenylamino- 1, 3, 5-triazin-2- yl) amino]-2, 2'-stilbenedisulfonic acid, and disodium salt: 1, 3-Bis propane, 1- (1-pyrenyl) (4-Trimethylammoniumphenyl)-6-phenyl-1, 3, 5-hexatrieneiodide:N-[3-(1, 5-Disulfonaphtyl)]-N'-[4-(2, 2, 6, and 6-tetramethylpiperidine-N-oxide) thiouurea, and disodium salt:N-Ethoxycarbonylmethyl-6-methoxyquinolinium promide:6-Methoxy-N-(3-sulfopropyl) quinolinium, monohydrate:5-or-6- (N-Succinimidyloxycarbonyl)-3' and 6 -- '-diacetylfluorescein:5-or-6-(N-Succiniumidyloxycarbonyl)-4' and 5 '-diamethyl-3', 6'-O -- O' - diacetylfluorescein: 5-or-6- 6-bis-dimethylaminoacridine: (N-Succiniumidyloxycarbonyl)-4' and 5 '-dichloro-3', 6'- O and O'-diacetylfluorescein:3 -- 9-aminoacridine: Nine - (4-diethylamino-methylb) utylamino)-3-chloro-7-methoxyacridine:1-anilinonaphthalene-8-sulfonate:N-methyl-2-anilinonaphthalene-6-sulfonate: 2-p-toluidinyl-naphthalene-6-sulfonate:12(9-anthroyloxy)stearic-acid:teramethyldiaminodiphenylketoimine hydrochloride:7-chloro-4-dimethylamino-1, 4, 4a, 5, 5a, 6 and 11, 12 a-octahydro -3, 6, 10 and 12, and 12 a-pentahydroxy:cyanine dyes: 1 1'-dihexyl-2, 2' - oxacarbocyanine: 3, 3'-dipropylthiadicarbocyanine:5-[(3-sulfopropyl-2(3H)-benzoxazolylidene) -2-butenylidene]-1, and 3-dibutyl-2-thiobarbituric acid: 5-dimethylaminonaphthalene-1-sulfonamide:dansylamino-ethyltriphosphate:1-(5-dimethylaminonaphthalene-1-sulfonamide)-3-N, N and -dimethyaminopropane:1-(5-dimethylaminonaphthalene-1-sulfonamido)-propane-3-trimethlammonium:(N-dansyl)-aminoalkyl- beta-D-galactopyranoside: Epsilon-dansyl-L-lysine : N6-ethennoadenosine: dansylphosphaidyl-ethanolamine:1 and 6-diphenyl- 1, 3, and 5-hexatriene:2' -- 4', 5', and 7'-tetrabromofluorescein:1 -- 2 7-diamino-9-phenylphenanthrium-10-ethyl-bromide:9-(o-carboxyphenyl)-6-hydroxy-3 H-xanthen-3-one:7-amino-3- pyrazolo (Beta-D-ribrofuranosyl) pyrimidine: (4, 3-d) 4-benzoylamido-4 -- '-aminostilbene-2-2' -disulfonate:1-acyl-2-[N- (4-nitrobenzo-2-oxa-1 --) 3-diazolylaminocaproyl]phosphatridylcholin e:beta-naphthyltriphosphat e:oxonol dye:bis[3-phenyl-5-oxoisoxazol-4-yl] pentamethineo xonol:bis[1 and 3-dibutylbarbituric-acid (5)] pentamethineoxonol: alpha(9, 11, 13, 15-cis.trans, trans, cis) octadecatetraenoic Acid:beta It all(s). 9, 11, 13, and 15-transoctadecateraenoic acid:perylene:N-phenyl-1-napthylamine:pyrene:2, 3-dimethyl-3, 7-diamino-5-phenylphenazium:4-phenylspro[furan-2 (3H), 1'-futalan] -3 and 3'-dione:o-phthalic dicarboxaldehyde:1-dimethylaminonaphthalene-5-sulfonyl chloride:flurorescien isothiocyanate: 7-chloro-4-nitrobenzo-2-oxal, 3-diazole:N-dansyl aziridine:5-(iodoacetoamidoethyl) amino-naphthalene-1-sulfonate : 5-iodoacetamido fluorescein:N- maleimide:N-(7-dimethylamino-4-methylcoumarynul) maleimide:N-(3-pyrene) maleimide:eosin-5-i (1-anilinonaphthyl-4) odoacetamide:fluorescein mercuty-acetate: 2-[4'(2"-iodoacetamido) pheyl] aminonaphthalene-6-sulfonic acid: [0076] (4) The observation approach of a microscope given in claims 1 and 2 or the above (1) thru/or any of (3) they are. [which is characterized by making irradiation time to the sample of two waves of light shorter than the life time of fluorescence of the molecule which the sample is dyed]

[0077] (5) The observation approach of a microscope given in claim 1 thru/or 3 or the above (1) thru/or any of (4) they are. [which is characterized by making it irradiate said two waves of light in a sample at coincidence]

[0078] (6) Said wavelength lambda 2 Before irradiating light, it is said wavelength lambda 1. The observation approach of a microscope given in claim 1 thru/or 3 or the above (1) thru/or any of (5) they are.

[which is characterized by making it the exposure of light completed]
[0079] (7) Said wavelength lambda 2 It is the above lambda 1 about the irradiation time of light. The observation approach of a microscope given in claim 1 thru/or 3 or the above (1) thru/or any of (6) they are. [which is characterized by making it longer than the irradiation time of the light of wavelength]
[0080] (8) after the exposure of the light of two wavelength is completed by the time of day which detects fluorescence -- it is -- having made -- the observation approach of a microscope given in claim 1 thru/or 3 or the above (1) thru/or any of (7) they are. [which is characterized by things]
[0081] (9) The observation approach of a microscope given in claim 1 thru/or 3 or the above (1) thru/or any of (8) they are. [which is characterized by using a molecule with the life time of fluorescence longer than 1ns for dyeing of a sample]
[0082] (10) The observation approach of a microscope given in claim 1 thru/or 3 or the above (1) thru/or any of (9) they are. [which is characterized by observing the dyed sample in the solution of pH 6-11.5]
[0083]
[Effect of the Invention] As mentioned above, according to the observation approach of the microscope of this invention, the microscope image of the sample which has high spatial resolving power is obtained by illuminating the dyed sample by the light of two or more wavelength.

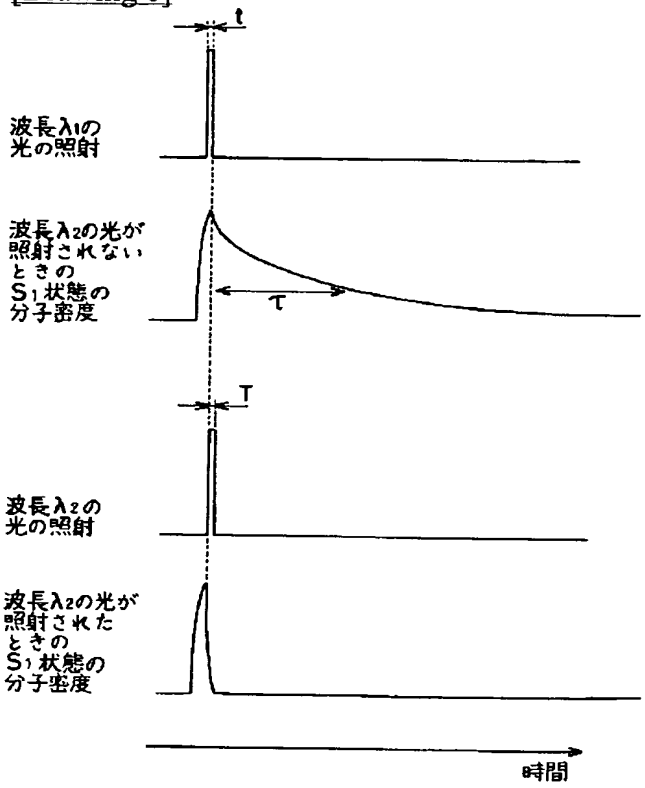
[Translation done.]

* NOTICES *

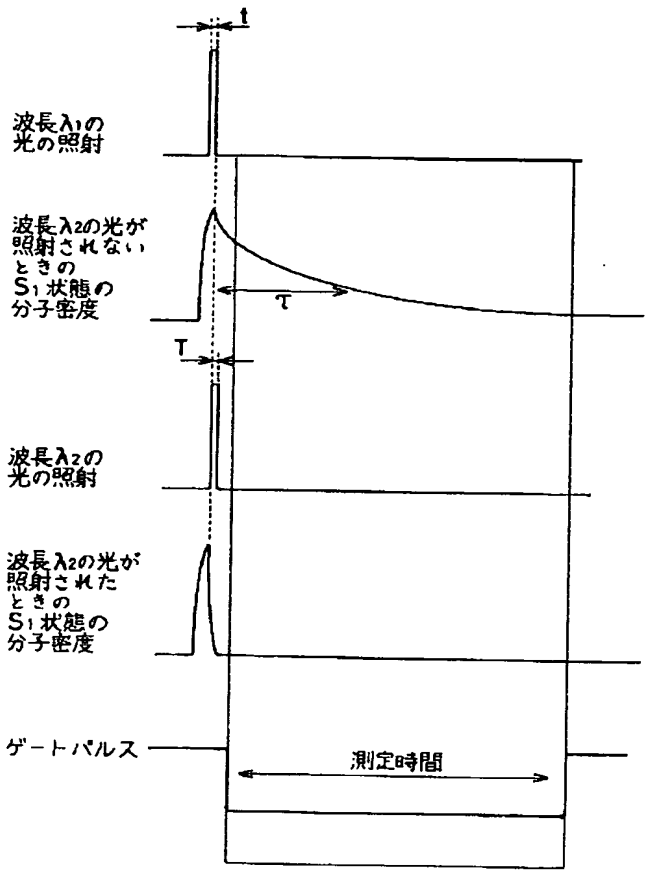
1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

DRAWINGS

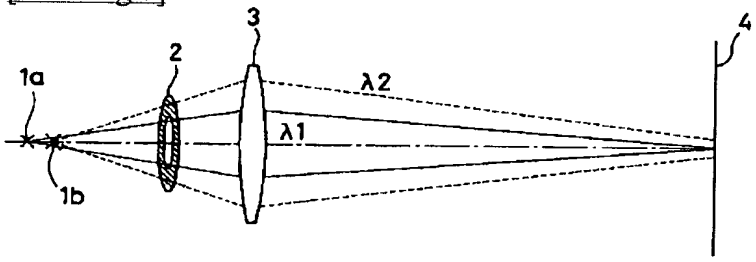
[Drawing 1]



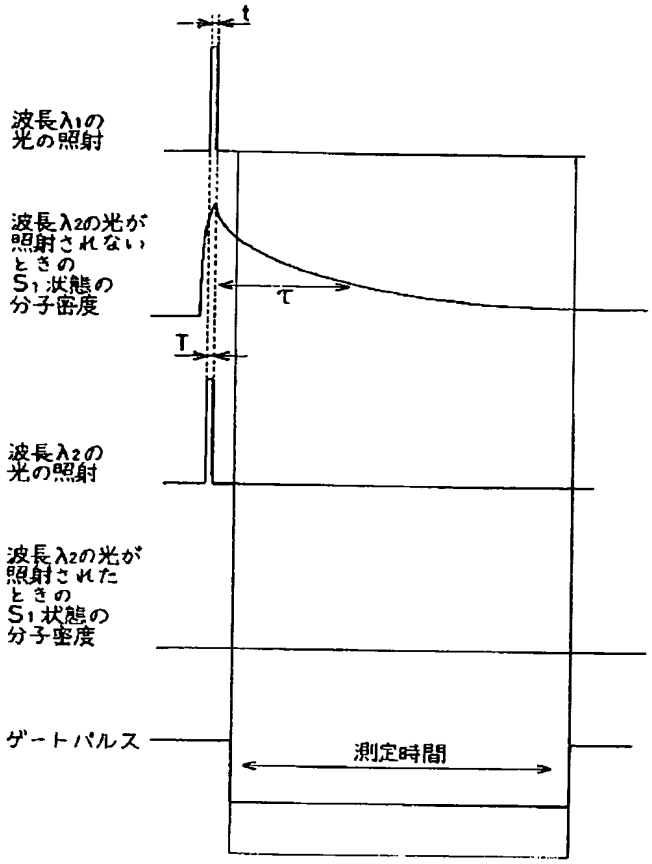
[Drawing 2]



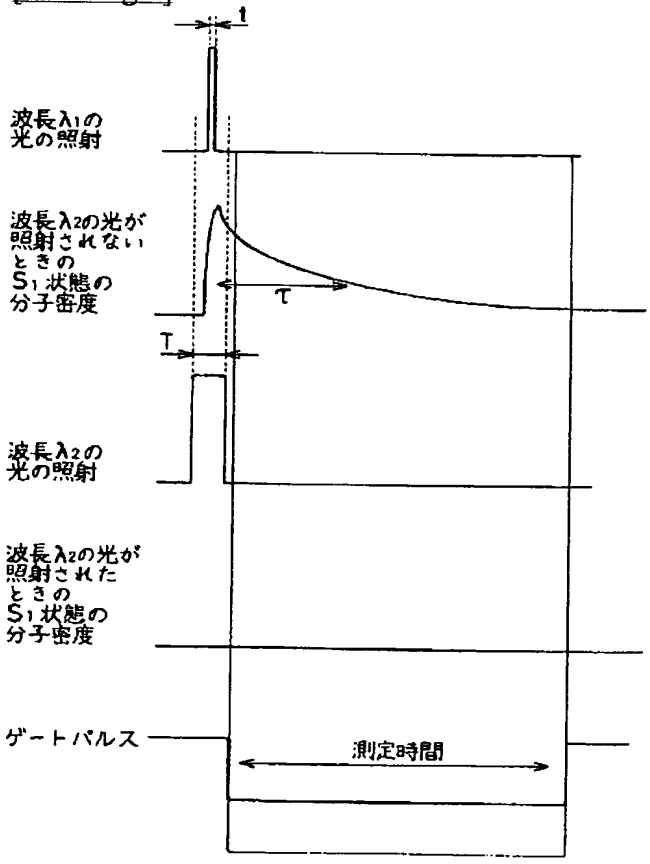
[Drawing 7]



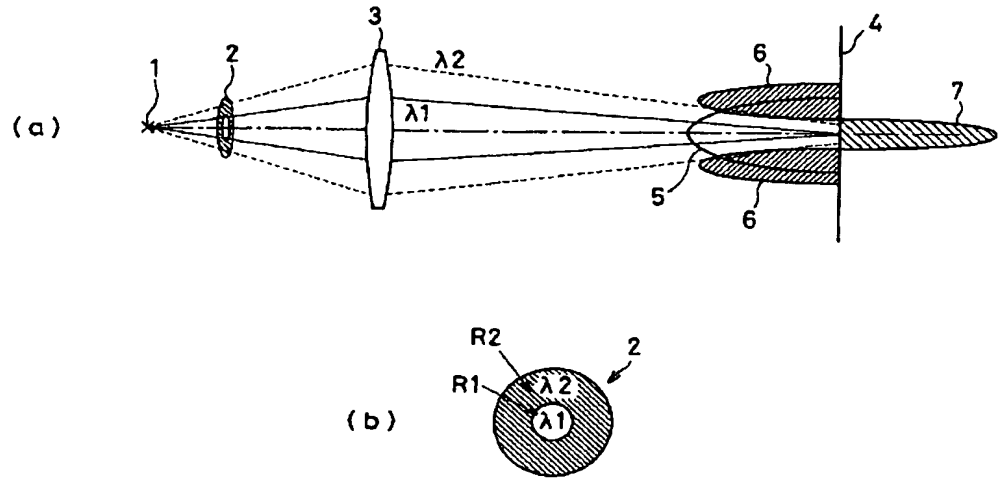
[Drawing 3]



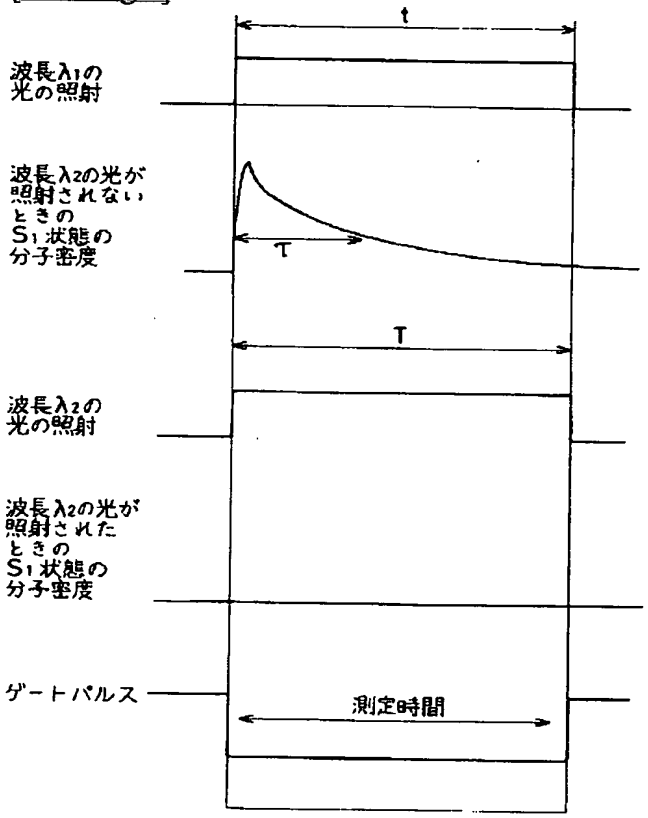
[Drawing 4]



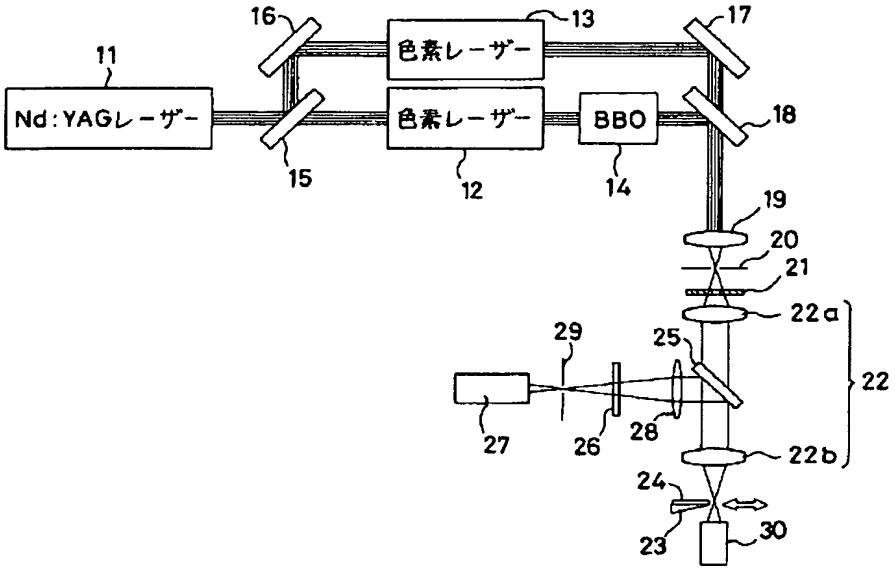
[Drawing 6]



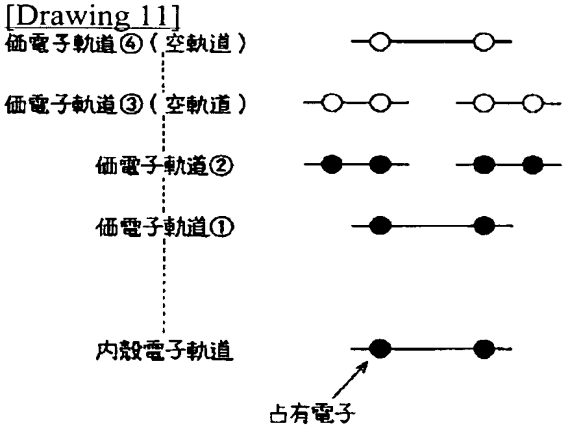
[Drawing 5]



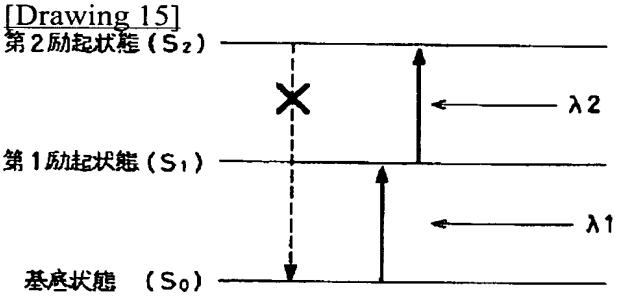
[Drawing 8]



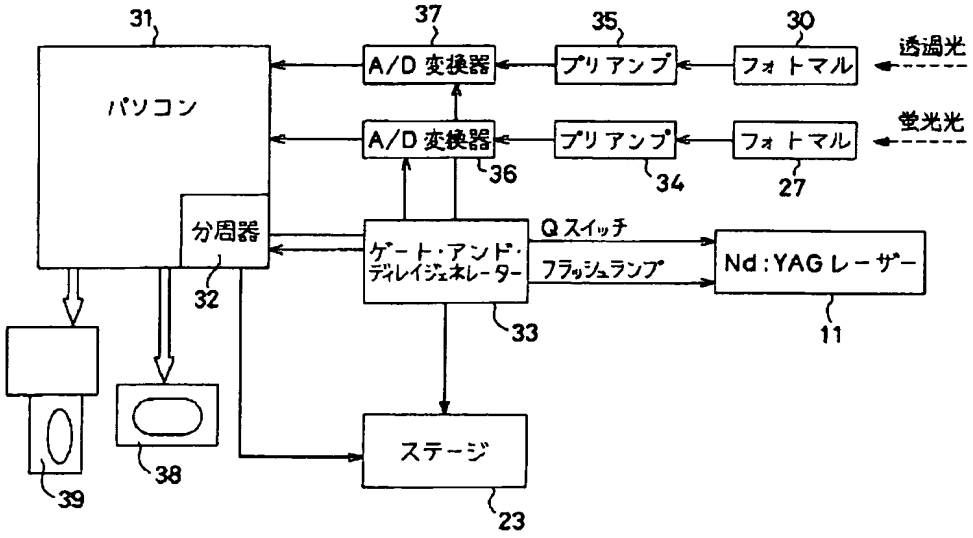
[Drawing 11]



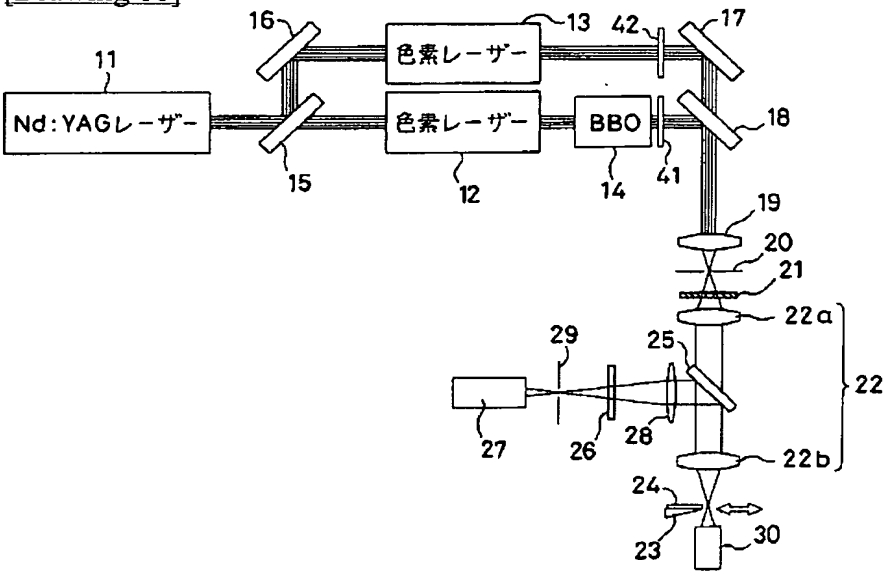
[Drawing 15]



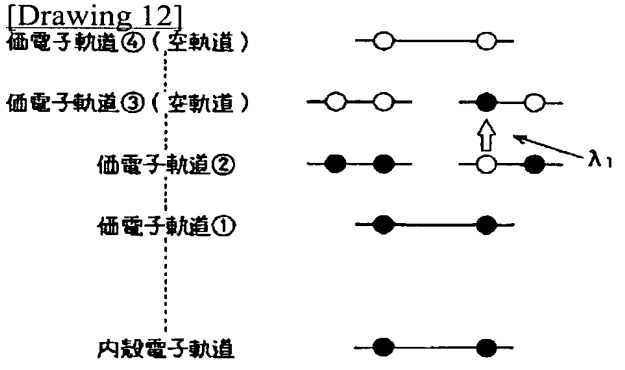
[Drawing 9]



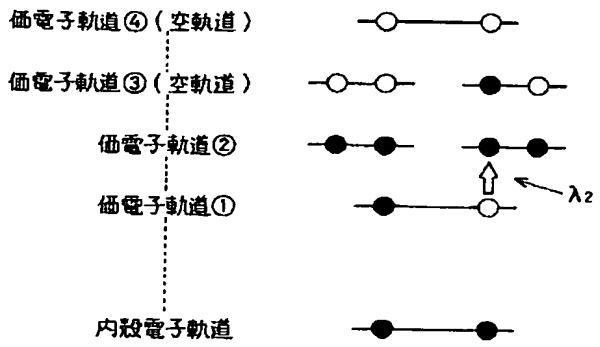
[Drawing 10]



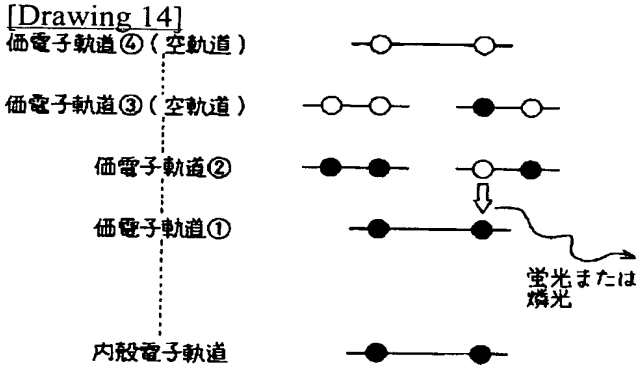
[Drawing 12]



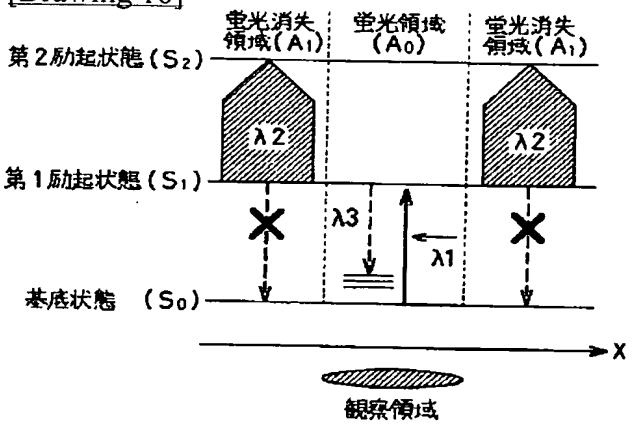
[Drawing 13]



[Drawing 14]



[Drawing 16]



[Translation done.]